oxygen levels in traditional hypoxic cell culture experiments may not accurately reflect *in vitro* ischemic environments. The oxygen tension within dental pulp tissue *in vivo* is difficult to measure directly. However, it is possible that the oxygen range commonly used in "hypoxic" cell culture experiments (2-5%) may not be an accurate approximation of *in vivo* conditions, and very low oxygen tension (less than 1%) might be necessary (Agata et al., 2008).

Experimental ischemia in dental pulp-derived cell culture

Several studies have demonstrated that low oxygen tension (2-5%) promotes proliferation of dental pulp-derived cells (Amemiya et al., 2003; Sakdee et al., 2007; Iida et al., 2010; Li et al., 2011). This finding may reflect the fact that dental pulp-derived cells are exposed to relatively low oxygen tension within their normal physiologic environment. When these cells are exposed to both hypoxia (2%) and serum deprivation for 24 or 48 hours, a condition that mimics *in vivo* ischemia, proliferation rates decline (Wang et al., 2010). Cells survive in this environment even though proliferation rates decline, suggesting a complex cellular defensive response to ischemia. This response includes suppression of cell growth and induction of cellular defense systems, including upregulation of hypoxia-inducible factor 1α (HIF-1α), heat shock protein 70 (HSP 70), and AMP-activated protein kinase (AMPK) (Amemiya et al., 2003; Fukuyama et al., 2007; Agata et al., 2008; Aranha et al., 2010). These factors may even activate cell growth after the ischemic event has resolved (Ueno et al., 2006; Fukuyama et al., 2007). This complex response to hypoxia and nutrient deprivation reflects a balance between damage due to noxious stimuli and activation of cellular defense systems.

The effect of low oxygen tension on the differentiation capacity of dental pulp-derived cells is unclear. After being cultured for 14 days in 5% O_2, dental pulp-derived cells increase expression of osteonectin (ON), dentin matrix protein-1 (DMP-1),

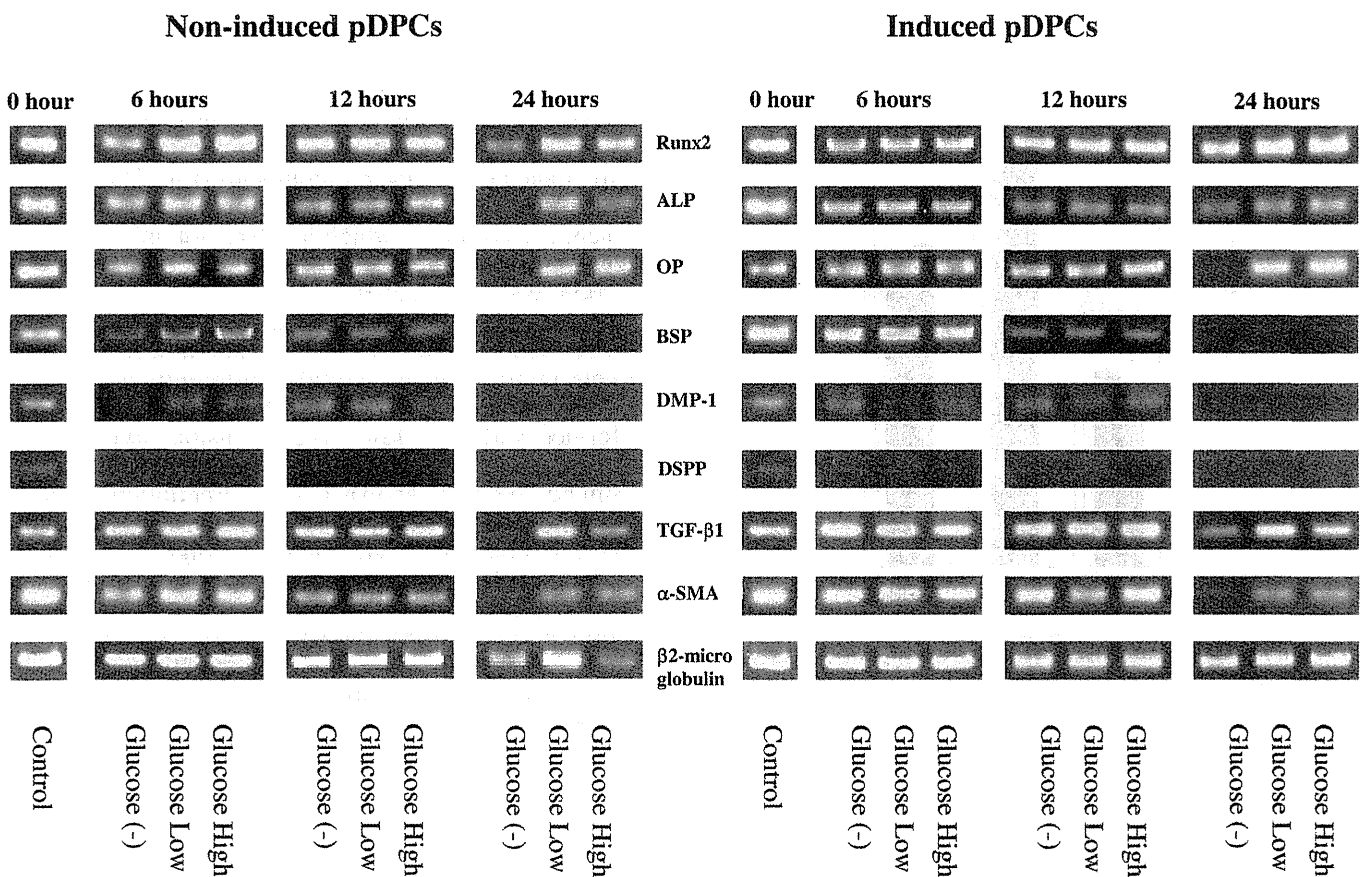


Fig. 1. Changes in the expression of odontogenic/osteogenic genes in non-induced and induced pDPCs cultured under severe-hypoxic conditions. Induced and non-induced pDPCs were cultured under severe hypoxic conditions in either low or high glucose concentration. Gene expression was analyzed by RT-PCR at 0, 6, 12 and 24 hours. Expression of Runx2, ALP, OP, TGFβ1, and α-SMA was slightly inhibited in both non-induced and induced cells. The expression of BSP, DMP-1, and DSPP was strongly inhibited in both populations. Non-induced and induced pDPCs underwent de-differentiation in a time-dependent manner. (From Agata et al., 2008 with modification).

bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP), and after 21 days significantly greater calcified nodule formation is observed. Both of these factors appear to promote differentiation (Li et al., 2011). Canine dental pulp-derived cells show decreased alkaline phosphatase activity (ALP, an early marker of osteogenic (odontogenic) differentiation) after 4 days in both hypoxic (2% O_2) and normoxic culture conditions, though ALP activity in the hypoxic cells remained higher than in the normoxic cells (Amemiya et al., 2004). Other studies have reported that hypoxia has an inhibitory effect on differentiation of dental pulp-derived cells. For example, we have demonstrated that porcine dental pulp-derived cells have significantly lower expression of BSP, DMP-1, and DSPP in both differentiation-induced and non-induced cells regardless of the severity of ischemia , though expression of other marker genes is not significantly different when cells are grown in glucose-containing medium (Fig. 1). Additionally, when human dental pulp cells are cultured under 3% O_2 for 14 days, ALP activity and the expression of DMP1, DSPP and osteocalcin are suppressed (Iida et al., 2010).

Investigations into the effect of low oxygen tension on the ability of dental pulp-derived cells to differentiate differ in terms of the species from which the cells were isolated and the conditions under which the cells were cultured (i.e. level and duration of hypoxia, and the status of cell differentiation), making direct comparisons difficult. However, accumulating evidence indicates that ischemic conditions do affect the differentiation of dental pulp-derived cells, and the type and magnitude of the effect may correlate with the severity of ischemia. Detailed cellular analyses are required to understand the mechanisms underlying this phenomenon.

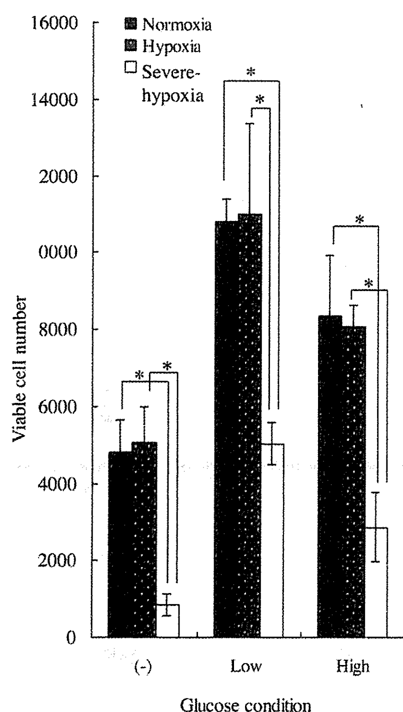


Fig. 2. Viability of porcine dental pulp-derived cells (pDPCs) in cell culture depends on ischemic conditions. To investigate the effect of differential ischemic conditions, pDPCs were cultured under a range of O_2 concentrations (normoxia, hypoxia, or severe-hypoxia) and glucose concentrations (high glucose, low glucose, or glucose (-)) in serum-free media. Viable cell number was calculated as the percentage of surviving cells. Significantly more cells survived under normoxic and hypoxic conditions than under severe-hypoxic conditions. No significant difference was observed between normoxic and hypoxic conditions. pDPCs cultured in low glucose media were significantly more viable than cells cultured in high glucose or glucose (-) media. Error bars represent the mean ± standard deviation for six separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

Characteristics of dental pulp-derived cells that survive under ischemic cell culture conditions.

Dental pulp tissue often recovers its function after an ischemic event, and ischemia-surviving cells likely contribute to tissue recovery. As previously discussed, traditional low oxygen culture conditions may not accurately reflect hypoxic environments *in vivo*. Accordingly, very low oxygen tension (<0.1% O_2, or even anoxia) is required for culture conditions to truly approximate physiologic environments (Fig. 2) (Agata et al., 2008). Furthermore, these cultures may require "ischemic conditions", which involves not only very low oxygen tension but also nutrient deprivation (Agata et al., 2008; Wang et al., 2010).

Investigations into the mechanisms by which dental pulp-derived cells recover from ischemia have focused on determining whether cellular differentiation affects survival. However, the number of experiments performed with very low oxygen tension (hypoxia) or ischemic conditions (hypoxia plus nutrient deprivation) is limited. We have shown that differentiation-induced and non-induced cells (possibly stem/progenitor cells) survive at equivalent rates under a range of ischemic conditions. Hence ischemia-tolerance is comparable between differentiated and non-differentiated cells (Agata et al., 2008). Despite these similarities, differences between the two cell populations do exist. Upregulation of the pluripotent stem cell markers octamer-binding transcription factor 4 (Oct4) and Sox2 is observed only in non-induced cells under ischemic conditions (Fig. 3). This finding suggests that ischemia-surviving non-odontogenic cells (undifferentiated cells) may be able to de-differentiate, acquiring greater growth and differentiation potential for post-ischemia tissue regeneration. In contrast, de-differentiation of ischemia-surviving odontogenic (differentiated) cells may allow them to re-acquire mitotic potential (pulp-resident odontoblasts are post-mitotic cells that are not able to divide or repair damaged dentin) (Liu et al., 2006).

Hence cells that survive ischemic insult may de-differentiate under ischemic conditions and obtain more stem cell-like characteristics, thereby contributing to the regeneration of damaged tissue.

Insights into the mechanisms of dental pulp tissue recovery after ischemic damage

Cellular damage caused by ischemia is generally irreversible even when the ischemic insult is transient. Dental pulp tissue can recover its functions after transient ischemia, suggesting that this tissue may undergo cellular regeneration rather than cellular recovery. The physiologic function of dental pulp (protection, nutrition, and tooth sensation) are supported by multiple cell types, hence post-ischemic pulp recovery has previously been attributed to multiple cell populations (Sloan and Smith, 2007). However, with the discovery of dental pulp stem cells (DPSCs) which are able to differentiate into multiple cell lineages (angiogenic, vasculogenic, neurogenic, chondrogenic, and osteo/odontogenic) a new paradigm has emerged in which a single stem cell population may be all that is required for pulp tissue recovery. Thus, it is of interest to investigate whether the post-ischemic recovery of pulp function is mediated solely by DPSCs.

One of the most important functions of dental pulp is to protect the tooth from noxious stimuli through dentin formation. Reparative dentin formation is frequently observed in teeth that survive ischemic insults (Spahr et al., 2002). The cell types responsible for this restorative process are unknown. Dental pulp contains terminally differentiated odontoblasts, but these cells are considered post-mitotic and thus not able to divide and form dentin (Liu et al., 2006). Additionally, odontoblasts may become necrotic under ischemic conditions. This has led to the assumption that reparative dentin formation following an ischemic event might be solely mediated by DPSCs (though other functions appear to be recovered by multiple cell populations) (About and Mitsiadis, 2001; Liu et al., 2006). However, as stated above, results from ischemic culture of induced dental pulp cells

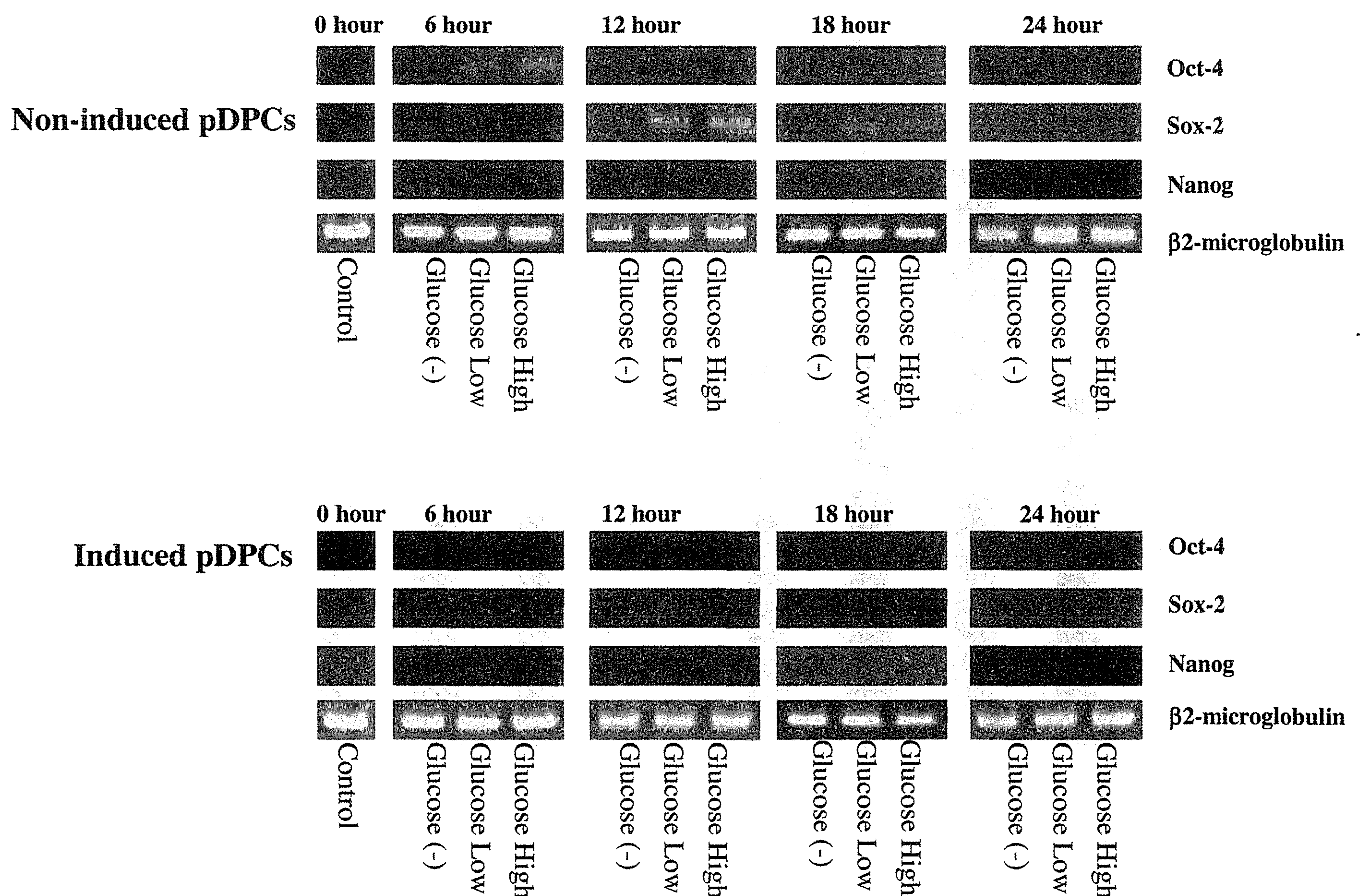


Fig. 3. Expression of pluripotent stem cell markers in induced and non-induced pDPCs cultured under severe-hypoxic conditions. RNA from induced and non-induced pDPCs cultured under severe-hypoxic conditions was extracted at 0, 6, 12, 18, 24 hours. Upregulation of the pluripotent stem cell markers Oct-4 and Sox-2 was observed only in non-induced pDPCs. Oct-4 was transiently activated at 6 hours, followed by the activation of Sox-2 from 12 to 18 hours. Nanog expression was not observed under any conditions. (From Agata et al., 2008 with modification).

(odontoblast-like cells) indicate that resident committed odontoblasts/precursors may de-differentiate and re-acquire mitotic potential during ischemia (Fig. 1). Therefore, post-ischemic reparative dentin formation may in fact be mediated by both DPSCs and de-differentiated odontoblasts, though further investigation is required to confirm this. If pulp-resident odontoblasts do contribute to post-ischemic reparative dentin formation, their ability to form dentin may be lower than that of DPSCs, because ALP activity of de-differentiated cells is lower in induced cells than in non-induced cells (which possibly contain DPSCs) (Fig. 4A,B).

Investigations into the post-ischemic recovery of other physiologic functions of dental pulp tissue are rare (e.g. nutrition and tooth sensation), but studies in other tissues demonstrate reparative roles for multiple cell types, including endothelial and neural cells (Sheridan and Bonventre, 2000; Bernert et al., 2003). Thus a more complete understanding of the mechanism of post-ischemic pulp tissue recovery should include analysis of endothelial and neural cell populations residing in dental pulp tissue. Finally, angiogenic factors, such as vascular endothelial growth factor (VEGF), are induced under hypoxic conditions, which may support the durability of dental pulp during hypoxia *in vivo* (Amemiya et al., 2003; Aranha et al., 2010).

Conclusion

It has long been known that dental pulp recovers its function after transient ischemia, but the mechanisms underlying this phenomenon have not been fully investigated, in part because *in vivo* monitoring of this tissue is anatomically difficult. Cell culture is emerging as a useful model for examination of cellular responses to ischemia and may become a valuable approach for investigation into the mechanisms of post-ischemic pulp tissue recovery.

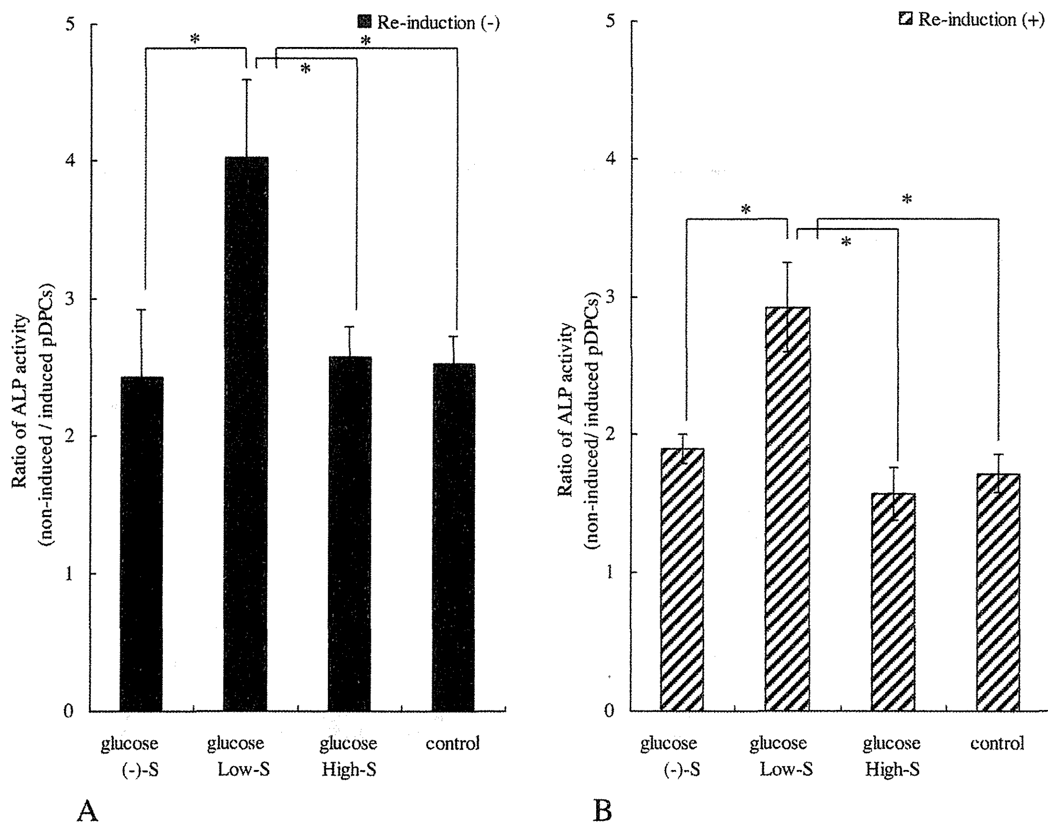


Fig. 4. Differentiation potential of odontogenic cells after ischemic de-differentiation. Induced and non-induced pDPCs were cultured in serum-free D-MEM at various glucose concentrations and exposed to severe-hypoxia for 24 hours. Surviving cells were then "re"-differentiated. Controls consisted of induced and non-induced pDPCs, respectively, cultured in serum containing D-MEM with high glucose under normoxia. ALP activity after "re"-differentiation was measured and compared with that before "re"-differentiation in both non-induced pDPCs groups (re-induction (-), **A**) and induced pDPCs groups (re-induction (+), **B**). Although ALP activity was increased after "re"-differentiation in both groups, the differentiation plasticity was observed to be higher among non-induced pDPCs groups (re-induction (-), **A**) than that of induced pDPCs groups (re-induction (+), **B**). S: Severe-hypoxia. Error bars represent the mean ± standard deviation for three separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

Acknowledgements. This work was supported in part by a Japanese Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science.

References

About I. and Mitsiadis T.A. (2002). Molecular aspects of tooth pathogenesis and repair: *in vivo* and *in vitro* models. Adv. Dent. Res. 15, 59-62.

Acosta D., Puckett M. and McMillin R. (1978). Ischemic myocardial injury in cultured heart cells: leakage of cytoplasmic enzymes from injured cells. In Vitro 14, 728-732.

Agata H., Kagami H., Watanabe N. and Ueda M. (2008). Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. Differentiation 76, 981-993.

Amemiya K., Kaneko Y., Muramatsu T., Shimono M. and Inoue T. (2003). Pulp cell responses during hypoxia and reoxygenation *in vitro*. Eur. J. Oral Sci. 111, 332-338.

Aranha A.M., Zhang Z., Neiva K.G., Costa C.A., Hebiling J. and Nor J.E. (2010). Hypoxia enhances the angiogenic potential of human dental pulp cells. J. Endod. 36, 1633-1637.

Bernert G., Hoeger H., Mosgoeller W., Stolzlechner D. and Lubec B. (2003). Neurodegeneration, neuronal loss, and neurotransmitter changes in the adult guinea pig with perinatal asphyxia. Pediatr. Res. 54, 523-528.

Das R., Jahr H., van Osch G.J. and Farrell E. (2010). The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue Eng. Part B Rev. 16, 159-168.

Fukuyama Y., Ohta K., Okoshi R., Suehara M., Kizaki H. and Nakagawa K. (2007). Hypoxia induces expression and activation of AMPK in rat dental pulp cells. J. Dent. Res. 86, 903-907.

Gong Q., Jian H., Wei X., Ling J. and Wang J. (2010). Expression of erthropoietin and erythropoietin receptor in human dental pulp. J. Endod. 36, 1972-1977.

Gronthos S., Mankani M., Brahim J., Gehron Robey P. and Shi S. (2000). Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. Proc. Natl. Acad. Sci. USA 97. 13625-13630

Gronthos S., Brahim J., Li W., Fisher L.W., Cherman N., Boyde A., Denbesten P., Robey P.G. and Shi S. (2002). Stem cell properties of human dental pulp stem cells. J. Dent. Res. 86, 531-535.

Iida K., Takeda-kawaguchi T., Tezuka Y., Kunisada T., Shibata T. and Tezuka K. (2010). Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells. Arch Oral Biol. 55. 648-654.

Iohara K., Zheng L., Ito M., Tomokiyo A., Matsushita K. and Nakashima M. (2006). The side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis and neurogenesis. Stem Cells 24, 2493-2503.

Jones S.M., Novak A.E. and Elliott J.P. (2011). Primary culture of cellular subtypes from postnatal mouse for *in vitro* studies of oxygen glucose deprivation. J. Neurosci. Methods 199, 241-248.

Li L., Zhu Y.Q., Jiang L., Peng W. and Ritchie H.H. (2011). Hypoxia promotes mineralization of human dental pulp cells. J. Endod. 37. 799-802.

Liu H., Gronthos S. and Shi S. (2006). Dental pulp stem cells. Methods Enzymol. 419, 99-113.

Lennon D.P., Edmison J.M. and Caplan A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. J. Cell Physiol. 187, 345-355.

Rodrigues C.A., Fernandes T.G., Diogo M.M., de Silva C.L. and Cabral J.M. (2010). Hypoxia enhances proliferation of mouse embryonic stem cell-derived neural stem cells. Biotechnol. Bioeng. 106, 260-270.

Sakdee J.B., White R.R., Pagonis T.C. and Hauschka P.V. (2009). Hypoxia-amplified proliferation of human dental pulp cells. J. Endod. 35, 818-823.

Sloan A.J. and Smith A.J. (2007). Stem cells and the dental pulp: potential roles in dentine regeneration and repair. Oral. Dis. 13, 151-157.

Sheridan A.M. and Bonventre J.V. (2000). Cell biology and molecular mechanisms of injury in ischemic acute renal failure. Curr. Opin. Nephrol. Hypertens. 9, 427-434.

Shinzawa K. and Tsujimoto Y. (2003). PLA2 activity is required for nuclear shrinkage in caspase-independent cell death. J. Cell. Biol. 163, 1219-1230.

Spahr A., Lyngstadaas S.P., Slaby I., Haller B., Boeckh C., Tsoulfidou F. and Hammarström L. (2002). Expression of amelin and trauma-induced dentin formation. Clin. Oral. Investig. 6, 51-57.

Sugiyama M., Iohara K., Wakita H., Hattori H., Ueda M., Matsushita K. and Nakashima M. (2011). Dental pulp-derived CD31?/CD146? side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats. Tissue Eng. Part A 17, 1303-1311.

Tsukamoto-Tanaka H., Ikegame M., Takagi R., Harada H. and Ohshima H. (2006). Histochemical and immunocytochemical study of hard tissue formation in dental pulp during the healing process in rat molars after tooth replantation. Cell Tissue Res. 325, 219-229.

Ueno Y., Kitamura C., Terashita M. and Nishihara T. (2006). Re-oxygenation improves hypoxia-induced pulp cell arrest. J. Dent. Res. 85, 824-828.

Wang J., Wei X., Ling J., Huang Y. and Gong Q. (2010). Side population increase after simulated transient ischemia in human dental pulp cell. J. Endod. 36, 453-458.

Accepted March 25, 2013

GROWTH FACTORS

http://informahealthcare.com/grf
ISSN: 0897-7194 (print), 1029-2292 (electronic)

Growth Factors, 2013; 31(5): 165–173
© 2013 Informa UK Ltd.
DOI: 10.3109/08977194.2013.830611

informa healthcare

ORIGINAL ARTICLE

GDFs promote tenogenic characteristics on human periodontal ligament-derived cells in culture at late passages

Dengsheng Xia[1,2], Yoshinori Sumita[2,3], Younan Liu[2], Yunlin Tai[2], Jinsong Wang[1], Masataka Uehara[3], Hideki Agata[4], Hideaki Kagami[4], Zhipeng Fan[1], Izumi Asahina[3], Songlin Wang[1], and Simon D. Tran[2]

[1]*Salivary Gland Disease Center and Molecular Laboratory for Gene Therapy & Tooth Regeneration, Capital Medical University School of Stomatology, Beijing, China,* [2]*Faculty of Dentistry, McGill University, Montreal, Canada,* [3]*Department of Regenerative Oral Surgery, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, and* [4]*Division of Molecular Therapy, The Institute of Medical Sciences, The University of Tokyo, Tokyo, Japan*

Abstract

Tendon/ligament injures are leading disabilities worldwide. The periodontal ligament (PDL) connects teeth to bone, and is comparable to a tendon/ligament-to-bone insertion. PDL-derived cells (PDLCs) express both osteo/cementogenesis and teno/ligamentogenesis genes. However, an efficient method to induce a tenogenic differentiation of PDLCs has not been thoroughly examined. Therefore, this study tested if growth/differentiation factors (GDFs) enhanced tenogenic characteristics of human PDLCs, as a potential cell source for tendon/ligament engineering. Results demonstrated recombinant GDF-5/GDF-7 inhibited alkaline phosphatase (ALP) activity of PDLCs from passage 3 to 6, while GDF-5 enhanced ALP in dental pulp-derived cells and mesenchymal stem cells. GDF-5 (particularly at 10 ng/ml concentration) induced high expression of both early (*scleraxis*) and mature (*tenomodulin, aggrecan, collagen3*) tenogenic genes in P4-6 PDLCs, while inhibiting expression of specific transcription-factors for osteogenic, chondrogenic and adipogenic differentiation. Exogenous GDFs might lead PDLCs being expanded in culture during several passages to highly useful cell source for tendon/ligament engineering.

Keywords

Growth differential factors, periodontal ligament cells, tenocyte differentiation, tissue engineering

History

Received 1 April 2013
Revised 17 July 2013
Accepted 28 July 2013
Published online 30 September 2013

For personal use only.

Introduction

Tendon and ligament injuries are leading disabilities for athletes and the active working population (Rodrigues et al., 2013). There is a sizeable economical burden associated with these medical injuries. Researchers in tissue engineering strive to cure these injuries by testing three critical components, which are the cell source, carrier vehicle (scaffold), and bioactive molecules (growth factors) for the repair/regeneration of tendons and ligaments (Gott et al., 2011). Our group has been working on obtaining an easily accessible autologous cell source for the engineering of tendon/ligament (Inoue et al., 2012). The periodontal ligament (PDL) is the tissue that connects teeth to bone, and can be compared to a tendon/ligament-to-bone insertion (Smith et al., 2012). Native periodontal ligament-derived cells (PDLCs) express both hard tissues (osteo- and cemento-genesis) and soft tissues (teno- and ligamento-genesis) genes. These cells can be easily obtained from common teeth removal procedures (such as wisdom teeth or for orthodontic purposes) and can be expanded *in vitro* in large quantities like tenocytes and dermal fibroblasts (the currently preferred and accessible cell source for engineering tendons). Current research on using PDLCs as a cell source has mainly focused on bone tissue engineering (Cabral et al., 2007; Chung et al., 2009; Hiraga et al., 2009; Ikeda et al., 2011; Saito et al., 2002; Seo et al., 2004; Shi et al., 2005) because PDLCs can be coaxed into mineralized tissues like the cementum and alveolar bone, with growth factors such as bone morphogenetic protein-2 (BMP-2) (Lin et al., 2013; Zhao et al., 2002). However, little is known about the bioactive molecules that can induce/enhance the tenogenic characteristics of PDLCs. In other words, while many studies induced the ostegogenic characteristics of PDLCs *in vitro* and *in vivo*, their induction into a tenogenic phenotype has not been well examined.

Growth/differentiation factors (GDFs) are a subfamily of the BMPs involved in skeletal and extra-skeletal tissues development (Chan et al., 1994; Merino et al., 1999; Storm et al., 1994; Storm & Kingsley, 1999; Thomas et al., 1996). GDFs, such as GDF-5, -6 and -7, are essential for normal skeletal development of the limb, including cartilage and joints (Erlacher et al., 1998; Faiyaz-Ul-Haque et al., 2002; Nakase et al., 2002; Settle et al., 2003; Stom & Kingsley, 1996). In addition to the osteo-inductive ability of traditional BMPs, the GDFs induce neo-tendon/ligament formation when implanted at ectopic sites *in vivo* (Wolfman et al., 1997).

Correspondence: Yoshinori Sumita, Assoc. Prof., 1-7-1 Sakamoto, Nagasaki 852-8588, Japan. Tel: +81-95-819-7701. Fax: +81-819-7705. E-mail: y-sumita@nagasaki-u.ac.jp
Simon D. Tran, Prof., 3640 University Street, M43, Montreal, Quebec, H3A 0C7, Canada. Tel: +1-514-398-7203. Fax: +514-398-2431. E-mail: simon.tran@mcgill.ca

Growth Factors, 2013; 31(5): 165–173

Therefore, GDFs have been considered as a marker for tendon/ligament tissues (Yoki et al., 2007). Indeed, numerous studies have demonstrated that GDFs can be used to enhance tendon and ligament regeneration in several animal models using different cell types and delivery materials (Bolt et al., 2007; Dines et al., 2007; Forslund et al., 2003; Park et al., 2010; Tan et al., 2012; Wolfman et al., 1997).

Expression of GDF-5, -6 and -7 mRNAs was detected during periodontal tissue formation (Morotome et al., 1998). Cells (expressing GDFs) were localized along the insertion sites of the periodontal ligament to the alveolar bone or cementum surfaces during root formation. GDFs expression was down-regulated in these cells after completion of root formation (Sena et al., 2003). Recently, our group demonstrated that GDF-5 also regulated differentiation of other cell types during (porcine) tooth development (such as dental papilla and dental follicle cells) and might affect differentiation co-operatively with other growth factors such as BMP-2 (Sumita et al., 2010). Meanwhile, rhGDF-7 was shown to form a functionally oriented periodontal ligament between newly formed bone and cementum (Miyoshi, 2002). In addition, recombinant human (rh) GDF-5 was demonstrated to enhance the proliferation of cultured human PDLCs and reduced alkaline phosphatase activity (ALP is a marker of osteoblastic activity) (Nakamura et al., 2003). Then, our group reported that recombinant mouse (rm) GDF-5 induced the expression of *scleraxis*, a crucial transcription factor of tenocytic differentiation, in cultured human PDLCs (Inoue et al., 2012). Taken together, these data suggest that GDFs can regulate periodontal ligament formation and that PDLCs could be a potential cell source for tendon engineering. However, there are selected studies reporting that GDFs induced osteo/cementogenesis in periodontal defects (Kim et al., 2009; Kwon et al., 2010; Lee et al., 2010; Stavropoulos et al., 2009).

Thus, more data are required to characterize GDFs' action on cultured PDLCs. During our work on bone engineering using PDLCs, we unexpectedly discovered that the expressions of osteogenic- and mature tenogenic-genes in PDLCs were severely decreased after passage 3 (P3) (Itaya et al., 2009). However, expression of *scleraxis* mRNA (a marker for early tendon formation/genesis) persisted in cultured PDLCs even after passage 6 (P6). This finding led us to believe that PDLCs could be induced toward a tenogenic differentiation at this specific "time window" (i.e. at passage 3 and higher). However, an efficient method to induce tenogenic differentiation on cultured PDLCs for the goal of tendon/ligament tissue regeneration has not been reported yet. Therefore, the aim of this study was to make progress towards establishing suitable cell culture conditions for the induction of PDLCs into tenocyte-like cells. Here, we analyzed the capacity of recombinant GDFs to enhance or to maintain tenogenic characteristics of human PDLCs being expanded in culture during several passages (P3 to P6) for potential use as a cell source for tendon/ligament engineering.

Materials and methods

Preparation of PDLCs

This study conformed to the tenets of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Capital Medical University School of Stomatology, Nagasaki University, and McGill University. Informed consent was obtained from each subject prior to donation of the tissue.

Normal healthy erupted third molars or premolars extracted for orthodontic reasons were collected from five patients (ages 18, 19, 21, 24 and 30 years; two males and three females). Each tooth sample was rinsed twice in a phosphate-buffered saline (PBS) solution containing 1000 units/ml of penicillin G sodium, 1 mg/ml streptomycin sulfate, and 2.5 μg/ml of amphotericin B (Invitrogen, Carlsbad, CA) for 5 min at room temperature (RT). Periodontal ligament (PDL) tissue was mechanically removed from the root surface by a scalpel. To avoid contamination by the gingival and apical regions, this coronal and apical PDL tissue was discarded. Tissue was digested with 2 mg/ml collagenase (Worthington Biochem, Freehold, NJ) for 1 h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Thermo Trace Ltd, Melbourne, Australia) and antibiotic-antimycotic solution on a shaker at 37 °C. A single cell suspension was obtained by filtering the cell suspension through a 70 μm strainer (Falcon, BD labware, Franklin Lakes, NJ). Then, after centrifugation for 5 min at 440 *g*, the cell pellet was re-suspended in culture medium (DMEM containing 10% FBS and antibiotic-antimycotic solution). Isolated cells were seeded into 10-cm culture dishes, and then incubated at 37 °C in 5% CO_2. When cultured cells reached 80–90% confluence, they were trypsinized and sub-cultured (1×10^5 cells in a 10-cm dish) until passage 2. Experiments in this study used cells at passage 3 (P3, referred previously by our group as cells in "early passages", P1 to P3; Itaya et al., 2009) and passages 4–6 (P4-6; referred as "late passages"; Itaya et al., 2009). Similarly, human dental pulp-derived cells (DPCs) and mesenchymal stem cells (MSCs) were cultured until passage 3 as controls. DPCs and PDLCs were harvested from the same teeth of patients, and MSCs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD).

Characteristic analysis of PDLCs

Flow-cytometry experiments used a FACscan argon laser cytometer (BD, San Jose, CA). Cells were harvested in 0.25% trypsin/EDTA and fixed for 30 min in ice-cold 2% formaldehyde. The fixed cells were washed in flow-cytometry buffer (PBS, 2% FBS, 0.2% Tween-20) and incubated for 30 min in flow-cytometry buffer containing fluorescein isothiocyanate-conjugated monoclonal antibodies to Stro-1 (R&D, Minneapolis, MN) and antigens CD34, CD45, CD90, CD105, CD146 and CD271 (Becton, Mountain View, CA).

To confirm the presence of Vimentin and Stro-1 proteins in PDLCs, immunofluorescence-cytostaining was performed. P5 PDLCs were cultured in a chamber slide until 70% confluent. The slides were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min. After incubation for 30 min with PBS containing 5% donkey (Jackson ImmunoResearch, Baltimore Pike, PA) or 5% goat (Vector Laboratories, Burlingame, CA) serum albumin as a blocking reagent and 0.25% Triton X-100, cells were incubated with mouse monoclonal anti-human Vimentin (1:50) (Dako, Via Real

For personal use only.

Carpinteria, CA) or mouse monoclonal anti-human Stro-1 (1:400) (Millipore, Temecula, CA) for 2 h at RT. The slides were then washed with PBS and incubated for 30 min at RT with FITC-conjugated donkey anti-mouse secondary antibody (1:200) (Jackson ImmunoResearch, Baltimore Pike, PA) and RHODAMINE-conjugated goat anti-mouse secondary antibody (1:100) (Millipore, Temecula, CA). After washing three times with PBS, slides were covered with a mounting reagent (Vector Laboratories, Burlingame, CA).

Treatments with GDF-5 and GDF-7

PDLCs at P3-P6 were seeded at concentration of 1×10^5 cells/dish into 10-cm dishes and were cultured in DMEM culture medium for 24 h. Then, the medium was replaced with DMEM supplemented with 1% FBS in the presence of recombinant mouse (rm) GDF-5 (R&D Systems, Minneapolis, MN) and/or recombinant human (rh) GDF-7 (BioVision, Mountain View, CA) at different concentrations (0, 10, 100 or 1000 ng/ml). These concentrations were chosen based on our preliminary experiments aiming to determine a range of GDFs concentrations that would reduce ALP activity of PDLCs in a dose-dependent manner (data not shown). Data reported in this paper were from GDFs at 10 or 100 ng/ml concentrations, and these concentrations were also chosen based on previous works by Park et al. (2010) and Inoue et al. (2012).

Total cell number and ALP activity analyses

Cell proliferation was measured using WST-8 kit (Dojindo, Kumamoto, Japan) according to manufacturer's protocol. Briefly, cells were incubated with medium containing 100 µl/ml of WST-8 for 1 h. The absorbance was read by a spectrophotometer at 450 nm (SmartSpeck™3000, BIO-RAD, Hercules, CA). ALP activities were measured according to the method of Lowry (1955). An aliquot of supernatant was added to p-nitrophenylphosphate containing $MgCl_2$ (Sigma-Aldrich, St. Louis, MO) and the mixture was incubated at 37 °C for 15 min. 0.2 N NaOH was added to stop the enzymatic reaction and absorbance was read at 415 nm with a spectrophotometer. ALP activity was expressed as µmol p-nitrophenol/cell. Cell proliferation and ALP activity were evaluated at 7 and 14 d after incubation with 0 and 100 ng/ml GDF-5 and/or GDF-7. Each experiment was performed in triplicate for three samples.

For ALP staining, GDF-5 treated PDLCs at day 14 of culture were fixed with 4% paraformaldehyde and stained with a solution of 0.25% naphthol AS-BI phosphate and 0.75% Fast Blue in an ALP kit, according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Signals were normalized based on protein concentrations (Fan et al., 2009).

Reverse transcription-polymerase chain reaction and quantitative PCR

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to determine the expression of tenogenic marker genes in P4 PDLCs cultured with GDF-5 and/or -7 (at 0, 10 and 100 ng/ml) for 7 d. Total cellular RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed on 2 µg of total RNA by SuperScript First-strand Synthesis (Invitrogen) according to the manufacturer's protocol. cDNA was amplified with Takara Taq (Takara Bio Inc., Shiga, Japan) and human-specific primer (*scleraxis, tenomodulin biglycan, collagen1, collagen3, gapdh*) sets are shown in the Appendix Table (online version only). Samples were incubated in a Thermal Cycler GP (Takara Bio Inc.) at 95 °C/(2 min) for 1 cycle and then 95 °C/(60 s), 56 °C/(60 s), and 72 °C/(60 s) for 20 cycles, with a final 5 min extension at 72 °C. After amplification, 10 µl of each product was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Furthermore, the effect of GDF-5 on the expression of tenogenic, osteogenic, chondrogenic and adipogenic mRNAs in P4-P6 PDLCs at 7, 14 and 21 d was evaluated by real-time fluorescent quantitative PCR. Real-time PCR reactions were performed with the QuantiTect SYBER Green PCR kit (Quagen, Hilden, Germany) and Icycler iQ Multi-color Real-time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA).The PCR consisted of an initial enzyme activation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Human-specific primer (*scleraxis, aggrecan, collagen3, tenomodulin, runx2, sox9, ppar*γ) sets used in this experiment are shown in the Appendix Table (online version only).

Statistics

One-way repeated measures ANOVA were used for the measurements of cell proliferation, ALP activity and quantitative gene expressions to detect any significant difference within each group. When a significant difference was detected, the differences among any selected groups were confirmed using Dunnett's test. Experimental values were presented as mean ± s.d. A p value of <0.05 was considered to be statistically significant.

Results

Characteristics of cultured PDLCs

PDLCs cultured at passage 4 had a characteristic spindle shape and formed a monolayer (Figure 1A). Immunofluorescence analysis revealed that human anti-Vimentin (mesenchymal cell marker) antibody reacted with cultured PDLCs (Figure 1B), and anti-Stro-1 (multi-lineage stem/progenitor marker) antibody reacted with some cells (Figure 1C). Cell surface markers on P4 PDLCs were characterized using immunofluorescence combined with flow-cytometric analysis. Many cells were positive for CD90 (86.28%) (mesenchymal cell marker), and there was a low level of CD105 (23.67%), CD146 (0.94%), CD271 (0.02%), and Stro-1 (2.18%) expressions (mesenchymal stem/progenitor cell markers). In contrast, expressions of CD34 and 45 (hematopoietic cell marker) was almost absent (less than 0.01%) (Figure 1D).

Effects of exogenous GDF-5 on cultured PDLCs

When P3 PDLCs were cultured with 100 ng/ml of GDF-5 for 14 d, their ALP activities were significantly lowered than that of cells cultured without GDF-5 (Figure 2A). In contrast,

For personal use only.

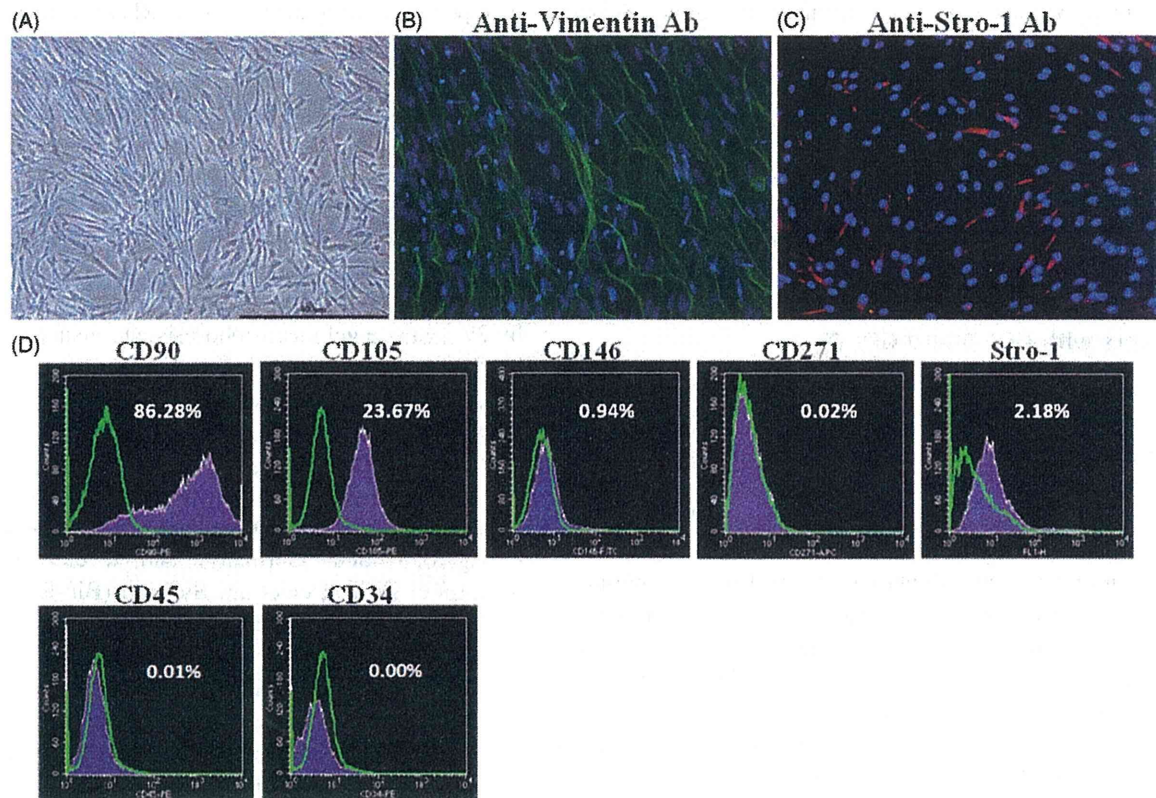


Figure 1. Cell characterization of cultured PDLCs at passage 4. (A) P4 PDLCs showed a characteristic spindle shape in DMEM with 10% FBS (40×). (B) Immunofluorescence staining of anti-Vimentin antibody. This mesenchymal cell marker is labeled by FITC (green); nuclei are labeled with DAPI (blue) (100×). (C) Immunofluorescence staining of anti-Stro-1 antibody. This stem cell marker (labeled by PE) (red) is detected in some cells; nuclei are labeled with DAPI (blue) (100×). (D) Flow cytometric analysis of mesenchymal stem cell markers (CD90, CD105, CD146, CD271, Stro-1) and hematopoietic cell markers (CD34, CD45) for P4 PDLCs.

For personal use only.

GDF-5 increased ALP activities of P3 DPCs (derived from same teeth with PDLCs) and MSCs, and this difference was significant between non-treated and GDF-5 treated DPCs (Figure 2A). To further confirm this inhibitory effect of GDF-5 on ALP activity of PDLCs, we performed cell staining and demonstrated that a dose-dependent increase in GDF-5 concentrations resulted in a decrease of ALP-positive P3 PDLCs cultured for 14 d (Figure 2B).

Then, we examined the effects of 100 ng/ml GDF-5 on P3 PDLCs and P4-6 PDLCs. Total cell number did not show a significant difference across all concentrations and passages at day 7 and 14 in culture (Figure 2C and E). However, GDF-5 exhibited a trend of inhibitory effect on ALP activity for all passages between P3-6 PDLCs at both day 7 and 14, with most significant differences on day 14 (Figure 2D and F); and in particular on P3 PDLCs cultured for 14 d.

Effects of GDF-5 and GDF-7 on ALP activity and tenogenic gene expressions

Next, the effect of GDF-7 on PDLCs was evaluated to confirm its induction potential for tenogenic differentiation. GDF-5 and/or GDF-7, at concentration of 100 ng/ml, were added to cultures of P4 PDLCs for 14 d. Again, total cell number did not significantly change across all groups at both day 7 and 14 (data not shown). On the other hand, GDF-7 reduced the ALP activity of P4 PDLCs as compared with non-treatment, but its inhibitory effect was less than that of GDF-5 (Figure 3A). When GDF-7 was combined with GDF-5, their synergistic effect exhibited the lowest level of ALP activity at 14 d in culture.

To further investigate the effect of exogenous GDF-5 and -7 on cultures, the expression of tenogenic marker genes was analyzed in P4 PDLCs treated with these proteins for 7 days. Expression of *scleraxis* (a transcription factor of early tendon formation), *biglycan*, *collagen 3* and *collagen 1* (extracellular matrix related genes in tendon) was observed in cells across all groups (Figure 3B). *Tenomodulin* mRNA (a mature tenocytic gene; a late marker for tendon formation) was not detectable in cells treated with GDF-7 alone. However, tenomodulin expression seemed up-regulated with GDF-5, and slightly up-regulated with both GDF-5 and -7 treatments.

Characteristic changes on PDLCs treated with GDF-5

Since GDF-7 did not demonstrate a significant effect on tenogenic expression (Figure 3B), we focused the remaining experiments on characterizing the effects of GDF-5 on the expression of several genes in P4-6 PDLCs cultured for 3 weeks. Results indicated a ~2-fold increase in the expression of *scleraxis* could be maintained by GDF-5 treatment at both 10 and 100 ng/ml for up to 21 d of culture (Figure 4A). The expressions of *aggrecan* and *collagen 3* (extracellular matrix proteins; used here as markers for mature tenocytes) were increased significantly in GDF-5 treated cells after 14 d of culture (Figure 4B and C). In particular, this tendency was remarkable (4- to 8-fold increase) in cells treated with 10 ng/ml of GDF-5. Moreover, PDLCs cultured with 10 ng/ml

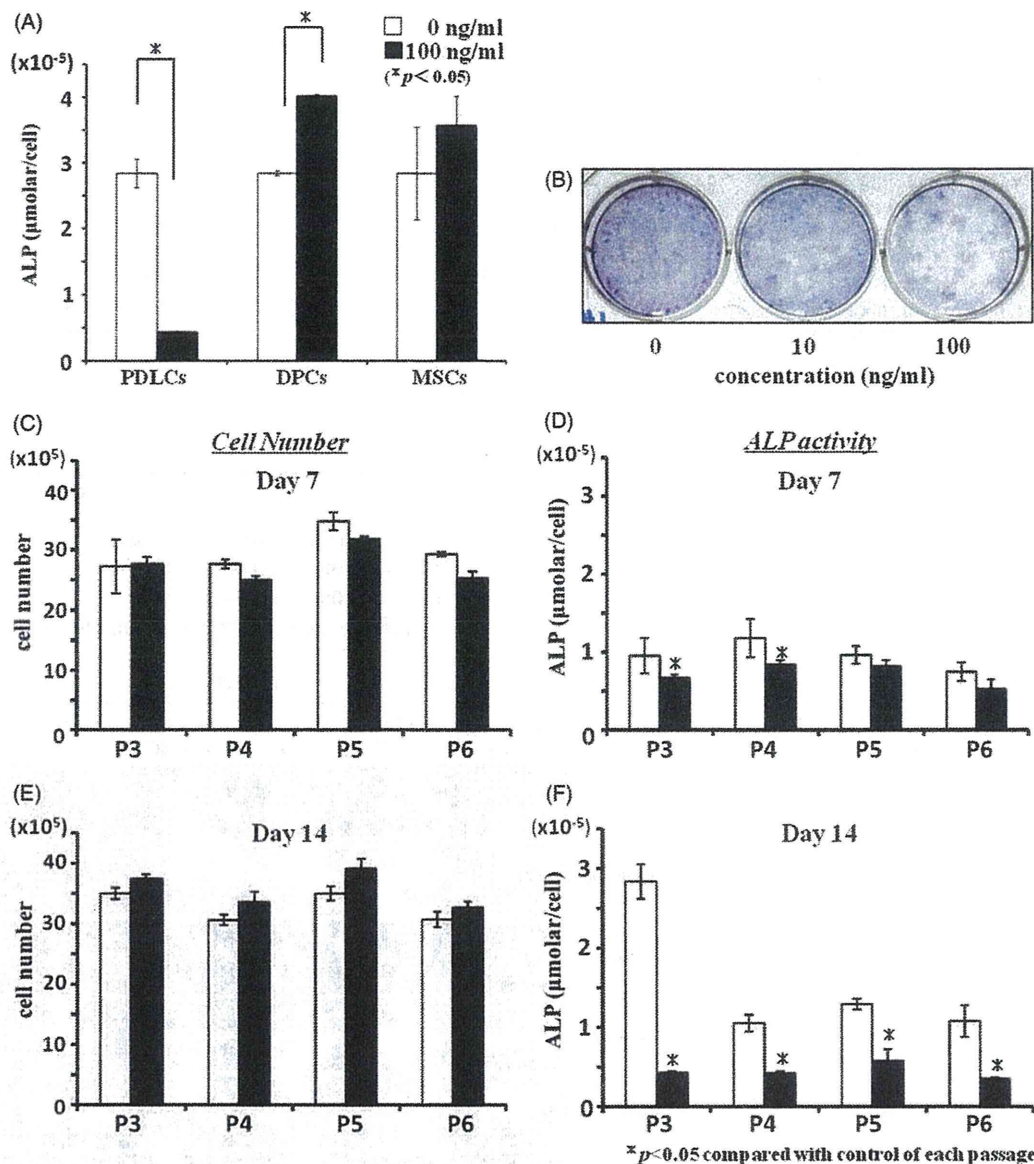


Figure 2. Effects of rmGDF-5 on different cell types. (A) Alkaline phosphatase (ALP) activity on P3 PDLCs, dental pulp-derived cells (DPCs) and mesenchymal stem cells (MSCs) at 14 d of culture. ALP activity in PDLCs decreased significantly by a treatment of 100 ng/ml GDF-5 (*$p<0.05$). In contrast, ALP activities of P3 DPCs and MSCs were increased by GDF-5 treatment with a statistically significant difference in DPCs. (B) GDF-5 treatment resulted in a reduced ALP staining of cultured PDLCs in a dose-dependent manner. (C, E) Total cell numbers for P3-6 PDLCs at 7 and 14 d of culture. Total cell number did not show a significant difference across all concentrations and passages. (D, F) ALP activities for P3-6 PDLCs at 7 and 14 d of culture. GDF-5 inhibited ALP activity for all passages between P3-6 PDLCs at both day 7 and 14. Most significant differences are shown on day 14 (particularly on P3 cells).

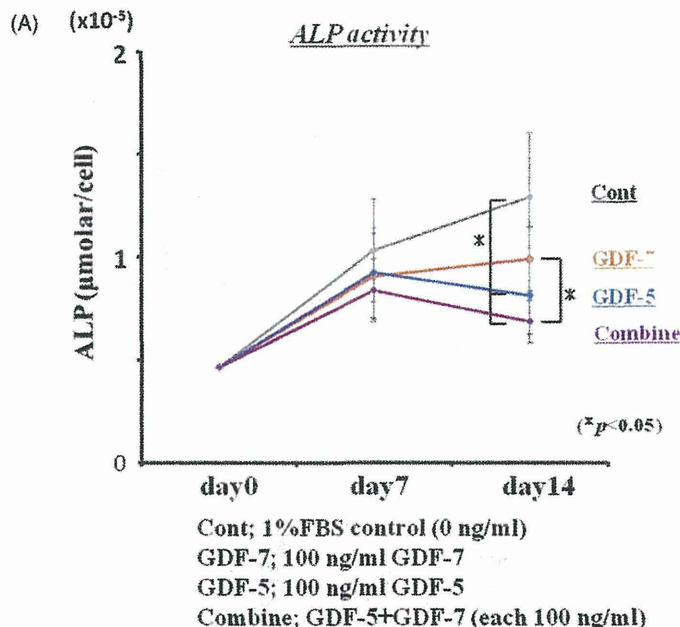
GDF-5 increased the expression of tenomodulin, a mature tenocyte marker, markedly at 21 d (Figure 4D). In contrast, no significant differences were observed for the expressions of *runx2* (a transcription factor of osteogenic differentiation), *sox9* (a transcription factor of chondrogenic differentiation) and *ppar*γ (a transcription factor of adipogenic differentiation) (Figure 4E–G).

Discussion

This study demonstrated that exogenous GDFs could reliably enhance the tenogenic/ligamental characteristics of primary cultured human PDLCs. Our outcomes were; (1) recombinant GDF-5 (and/or -7) maintained an inhibitory effect to ALP activity in PDLCs during several passages in culture, and (2) GDF-5 induced high expressions of both early and mature tenogenic genes in PDLCs cultured at late passages, while not enhancing the expressions of specific transcription-factors for osteogenic, chondrogenic and adipogenic differentiations.

Regarding the first outcome, we demonstrated that recombinant GDF-5 and -7 inhibited ALP activity reliably in cultured PDLCs. Our results may be consistent with Nakamura et al. (2003) who demonstrated that GDF-5 stimulated cell proliferation and glycosaminoglycan synthesis, but inhibited ALP activity in cultured PDLCs. However, a detailed characterization of changes in PDLCs treated with GDFs has not been reported. Thus our study confirmed that this inhibitory effect was persistent from passage 3 to 6. During the culture process, we observed that PDLCs had a tendency to down-regulate their osteogenic and tenogenic characteristics (Itaya et al., 2009). In fact, our data showed that the ALP activity of PDLCs after passage 4 was remarkably decreased when compared with that

For personal use only.



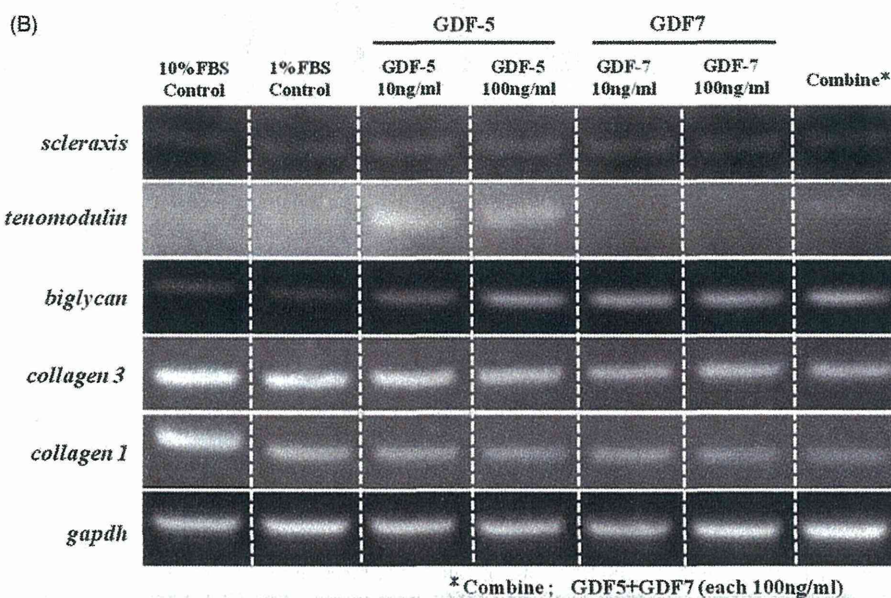


Figure 3. Effects of rmGDF-5 and rhGDF-7 on ALP activity and tenogenic gene expressions. (A) Effects of 100 ng/ml GDF-5 and/or GDF-7 treatments on the ALP activity of P4 PDLCs. GDF-7 reduced ALP activity as compared with non-treatment, but its inhibitory effect was less than that of GDF-5 at 14 d of culture. Combination of both GDFs resulted in the lowest level of ALP activity after 14 d of culture. (B) Changes of tenogenic gene expressions in P4 PDLCs by treatments with GDFs for 7 d in culture. RT-PCR products corresponding to *scleraxis*, *tenomodulin*, *biglycan*, *collagen 3* and *collagen 1* mRNAs. *Gapdh* was used as a loading control.

of cells at passage 3 (Figure 2F). Therefore, the inhibitory effect of GDF-5, alone, at P3 was significant because if these PDLCs P3 cells were not treated with GDF-5, their ALP activities would have remained high (i.e. a high ability of osteoblastic differentiation). GDF-5 was capable to suppress ALP activity to the same levels as that of PDLCs at passage 4–6 (Figure 2F). Moreover, we found recombinant GDF-7 has also an inhibitory effect on ALP activity of PDLCs although its effect was less than that of GDF-5, and that of both GDFs combined. Although the exact role/mechanism of GDFs during periodontal ligament formation has not been described, there are reports that GDFs were expressed in cells within the developing periodontal ligament (Morotome et al., 1998; Sena et al., 2003). And, this finding suggests GDFs to be involved in the formation of the dental-to-bone insertion/attachment apparatus. GDF-5 and -7 might play vital roles in inhibiting calcifications at insertion sites (borders) between bone/cementum and PDL.

In contrast to the inhibitory effect on ALP activity of PDLCs, GDF-5 had enhancing effects on DPCs or MSCs, interestingly. Similarly, we have previously shown that GDF-5 significantly reduced ALP activity in dental follicle-derived cells, while it significantly increased this activity in dental papilla-derived cells (Sumita et al., 2010). These observed differential effects of GDF-5 could depend on the cell types and their specific differentiation fates. GDF-5 promoted ALP activity in the osteo/odontogenic cells such as MSCs, DPCs or dental papilla-derived cells, while inhibited ALP activity in the tenogenic/ligamental cells such as PDLCs or dental follicle-derived cells. In particular, DPCs are believed to be more mature progenitors of osteo/odontoblastic cells. From these results, we speculate that the differential effects of GDF-5 on these cell types might be essential for the final development into bone/dentin or PDL. Therefore, the inhibitory effect of ALP activity displayed by GDFs might be extremely favorable to enhance or to induce the tenogenic characteristics of PDLCs.

With regard to the reliability of enhancing tenocytic induction in cultured PDLCs, our results indicated that GDF-5 strongly induced the expressions of both early and mature

For personal use only.

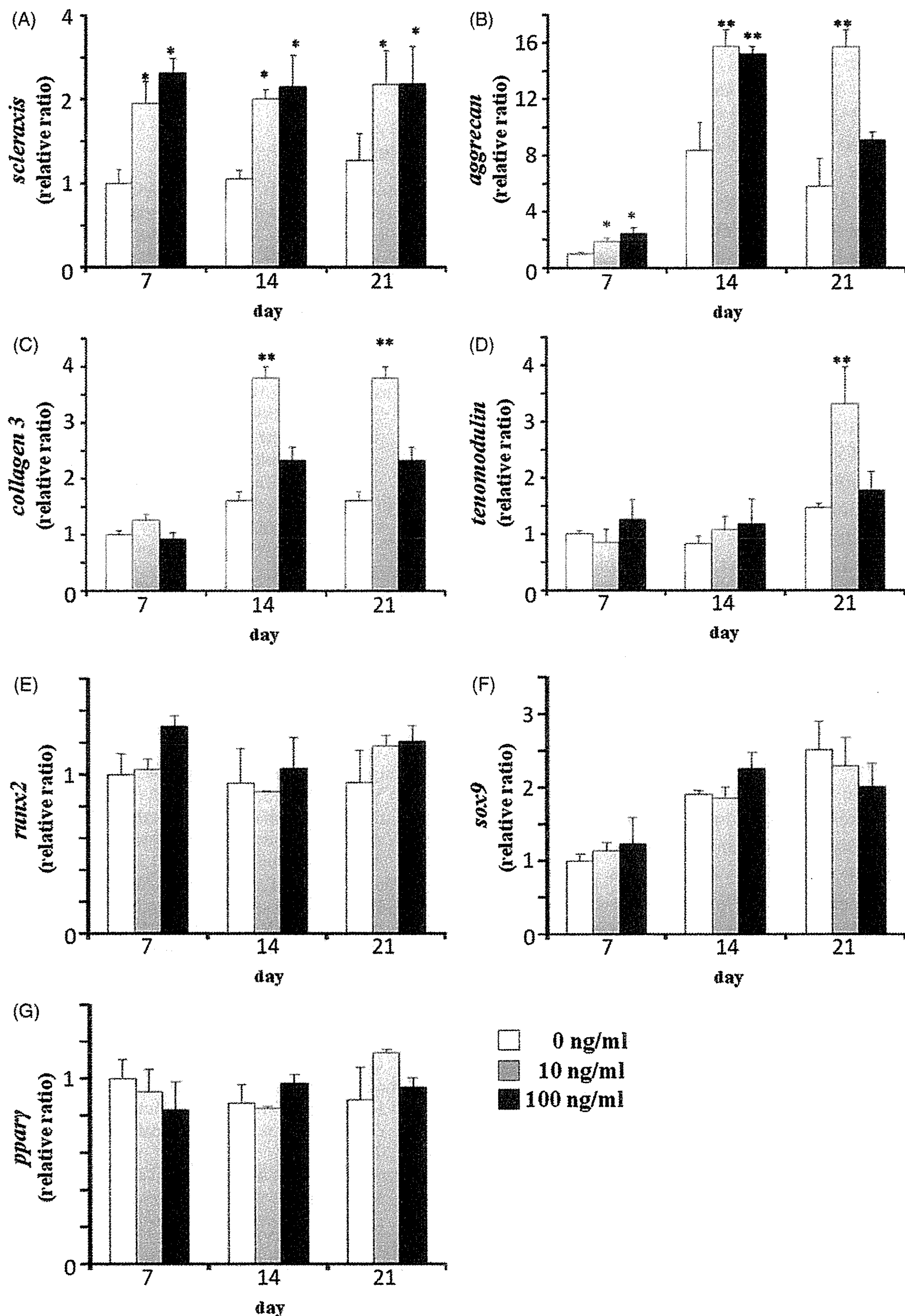


Figure 4. Characteristic changes on P4-6 PDLCs cultured with GDF-5 for 3 weeks. (A–D) Expression levels of *scleraxis* (A), *aggrecan* (B), *collagen 3* (C) and *tenomodulin* (D) mRNAs. (E–G) Expression levels of specific transcription-factors for osteogenic (*runx2*) (E), chondrogenic (*sox9*) (F) and adipogenic (*pparγ*) (G) differentiation. The asterisks represent statistical significance (*$p < 0.05$ and **$p < 0.01$) between the control (cells treated by 0 ng/ml) and the interests (cells treated by 10 and 100 ng/ml).

tenogenic genes, without enhancing the expression of osteogenic, chondrogenic or adipogenic genes. Human PDLCs cultured with GDF-5 showed high expression levels of *scleraxis* from the early phase of culture (7 d). Then, during late phases of culture (14–21 d), PDLCs expressed mature tenocytic *m*RNAs such as *tenomodulin*, *collagen 3*, and *aggrecan*. In particular, PDLCs cultured with 10 ng/ml of GDF-5 maintained high expression levels of these mature tenocytic *m*RNAs when compared with concentrations of 0 and 100 ng/ml GDF-5. For tendon formation, it is known that an inductive interaction between the myotome and sclerotome layers generate the syndetome, demarcated at the earliest stages of development by expression of the basic helix-loop helix transcription-factor gene, *scleraxis* (Towler & Gelbeman, 2006). Therefore, early expression of scleraxis has been considered to be a highly specific marker for

For personal use only.

tenogenic differentiation of mesenchymal progenitors (Park et al., 2010; Inoue et al., 2012; Schweitzer et al., 2001). In cultured PDLCs, our previous study demonstrated that *scleraxis* persisted at about the same levels during passages 1 to 6, while the expressions of mature tenogenic genes, such as *tenomodulin* or *collagen 12*, decreased gradually during passages (Itaya et al., 2009). Results from this study showed that GDF-5 treated PDLCs maintained approximately a 2-fold increase in the expression of *scleraxis* for over 3 weeks of culture, when compared with non-treated PDLCs. This reinforced expression of *scleraxis* in PDLCs might have induced the increased expression of *tenomodulin*, a mature tenogenic gene, at late phase of culture. It has been reported that *scleraxis* can upregulate *tenomodulin* expression in tenocytic progenitor cells (Park et al., 2010; Shukunami et al., 2006). PDLCs treated by GDF-5, especially 10 ng/ml in concentration, expressed a 2–3-fold increase in *collagen 3* or *aggrecan* at late phase of culture. This result indicated that GDF-5 might be able to directly enhance the production of these extracellular matrix (ECM) components of tendon fiber from PDLCs. Actually, tendon tissue is composed of tenocytes, collagen fibrils and ECM components (Park et al., 2010). Regarding such components, it has been suggested that collagen 3 has a role in endotenon and epitenon formation (Wang et al., 2005), and aggrecan, a proteoglycan of tendon ECM components, is localized to the compressed segments of tendons (Park et al., 2010; Rees et al., 2000). These data demonstrated that proper concentration of GDF-5 protein could induce a tenocytic differentiation of PDLCs accompanied with increased collagen and proteoglycan production in culture. In contrast, the significant changes of mRNA expressions in cells treated by GDF-7 were not observed in this study. It is known that GDF-7 does not induce bone or cartilage formation, and subcutaneous GDF-7 implantation induced the formation of tendon-like tissues in a rat model (Wang et al., 2005). Although the functions of GDF-7 on cultured PDLCs are not clear in this study, the role of this factor must be involved in the tenogenic differentiation of PDLCs. At least, as mentioned above, GDF-7 cooperates with GDF-5 for the inhibition of osteogenic differentiation in cultured PDLCs.

In conclusion, our findings strongly indicated that supplementation of GDFs in culture could induce periodontal ligament cells into a highly useful cell source for tendon/ligament tissue engineering. In addition, these PDLCs may regenerate both the periodontal ligament tissue as well as tendons/ligaments. Additional investigations will be needed to understand the practical usefulness of PDLCs and GDFs for reliable tendon/ligament tissue engineering.

Declaration of interest

This work was supported by grants from the National Natural Science Foundation of China (81141010 to D.S. Xia), the National Basic Research Program of China (No. 2010CB944801) and the Grant from Beijing Municipal Science and Technology Commission (Z121100005212004), and partially supported by the Grand-in-Aid for Scientific Research (22390375) from Japan Society for the Promotion of Science and Canada Research Chairs, Natural Sciences and Engineering Research Council (NSERC) of Canada. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Bolt P, Clerk AN, Luu HH, Kang Q, Kummer JL, Deng ZL, Olson K, et al. 2007. BMP-14 gene therapy increase tendon tensile strength in a rat model of Achilles tendon injury. J Bone Joint Surg Am 89: 1315–1320.

Cabral M, Costa MA, Fernandes MH. 2007. In vitro models of periodontal cells: Comparative study of long term gingival, periodontal ligament and alveolar bone cell cultures in the presence of beta-glycerophosphate and dexamethasone. J Mater Sci Mater Med 18:1079–1088.

Chan SC, Hoang B, Thomas JT, Vukicevic S, Luten FP, Ryba NJ, Kozak CA, et al. 1994. Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 269:28227–28234.

Chung IH, Yamaza T, Zhao H, Choung PH, Shi S, Chai Y. 2009. Stem cell property of postmigratory cranial neural crest cell their utility in alveolar bone regeneration and tooth development. Stem Cells 27: 866–877.

Dines JC, Weber L, Razzano P, Prajapati R, Timmer M, Bowman S, Bonasser L, et al. 2007. The effect of growth differentiation factor-5-coated sutures on tendon repair in a rat model. J Shoulder Elbow Surg 16:S215–S221.

Erlacher L, Ng CK, Ullrich R, Krieger S, Luyten FP. 1998. Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement *in vitro*. Arthritis Rheum 41: 263–273.

Faiyaz-Ul-Haque M, Ahmad W, Wahab A, Haque S, Azim AC, Zaidi SH, Teebi AS, et al. 2002. Frameshift mutation in the cartilage-derived morphogenetic protein 1 (CDMP1) gene and sever acromesomelic chondrodysplasia resembling Grebe-type chondrodysplasia. Am J Med Genet 111:31–37.

Fan Z, Yamaza T, Lee JS, Yu J, Wang SL, Fan G, Shi S, Wang CY. 2009. BCOR regulates mesenchymal stem cell function by epigenetic mechanisms. Nat Cell Biol 11:1002–1009.

Forslund C, Rueger D, Aspenberg P. 2003. A comparative dose-response study of cartilage-derived morphogenetic protein (CDMP)-1, -2 and -3 for tendon healing in rats. J Orhop Res 21:617–621.

Gott M, Ast M, Lane LB, Schwartz JA, Chatanzano A, Razzano P, Grande DA. 2011. Tendon phenotype should dictate tissue engineering modality in tendon repair: A review. Discov Med 12:75–84.

Hiraga T, Ninomiya T, Hosoya A, Takahashi M, Nakamura H. 2009. Formation of bone-like mineralized matrix by periodontal ligament cells in vivo: A morphological study in rats. J Bone Miner Metab 27: 149–157.

Ikeda H, Sumita Y, Ikeda M, Ikeda H, Okumura T, Sakai E, Nishimura M, Asahina I. 2011. Engineering bone formation from human dental pulp- and periodontal ligament-derived cells. Ann Biomed Eng 39: 26–34.

Inoue M, Ebisawa K, Itaya T, Sugito T, Yamawaki-Ogata A, Sumita Y, Wadagaki R, et al. 2012. Effect of GDF-5 and BMP-2 on the expression of tendo/ligamentogenesis-related markers in human PDL-derived cells. Oral Dis 18:206–212.

Itaya T, Kagami H, Okada K, Ymawaki A, Narita Y, Inoue M, Sumita Y, Ueda M. 2009. Characcteristic changes of periodontal ligament-derived cells during passage. J Periodontal Res 44:425–433.

Kim TG, Wikesjo UM, Cho KS, Chai JK, Pippig SD, Siedler M, Kim CK. 2009. Periodontal wound healing/regeneration following implantation of recombinant humam growth/differentiation factor-5 (rhGDF-5) in an absorbable collagen sponge carrier into one-wall intrabony defects in dogs: A dose-range study. J Clin Periodontol 36:589–597.

Kwon DH, Bennett W, Herberg S, Bastone P, Pippig S, Rodriguez NA, Susin C, Wikesjo UM. 2010. Evaluation of an injectable rhGDF-5/PLGA construct for minimally invasive periodontal regenerative procedures: A histological study in the dog. J Clin Periodontal 37: 390–397.

Lee JS, Wikesjo UM, Jung UW, Choi SH, Pippig S, Siedier M, Kim Ck. 2010. Periodontal wound healing/regeneration following implantation of recombinant human growth/differentiation factor-5 in a

For personal use only.

beta-tricalcium phosphate carrier into one-wall intrabony defects in dogs. J Clin Periodontol 37:382–389.

Lin Z, Rios HF, Park CH, Taut AD, Jin Q, Sugai JV, Robbins PD, Giannobile WV. 2013. LIM domain protein-3 (LMP3) cooperates with BMP7 to promote tissue regeneration by ligament progenitor cells. Gene Ther 20:1–6.

Lowry OH. 1955. Micromethods for the assay of enzyme. II. Specific procedures. Alkaline phosphatase. Methods Enzymol 4:371–372.

Merino R, Macias D, Ganan Y, Economides AN, Wang X, Wu Q, Stahl N, et al. 1999. Expression and function of Gdf-5 during digit skeletogenesis in the embryonic chick leg bud. Dev Biol 206:22–45.

Miyoshi Y. 2002. The expression of growth and differentiation factor (GDF)-7 gene during the development and remodeling of the hard tissues. Shikoku Dent Res 15:139–153.

Morotome Y, Goseki-Sone M, Ishikawa I, Oida S. 1998. Gene expression of growth and differentiation factor-5, -6, -7 in developing bovine tooth at the root forming stage. Biochem Biophys Res Commum 244: 85–89.

Nakamura T, Matuo Y, Tamura M, Izumi Y. 2003. Effects of growth/differentiation factor-5 on human periodontal ligament cells. J Periodont Res 38:597–605.

Nakase T, Sugamoto K, Miyamoto T, Tsumaki N, Luyten FP, Inui H, Myoui A, et al. 2002. Activation of cartilage-derived morphogenetic protein-1 in torn rotator cuff. Clin Orthop Relat Res 399:140–145.

Park A, Hogan MV, Kesturu GS, James R, Balian G, Chhabra AB. 2010. Adipose-derived mesenchymal stem cells treated with growth differentiation factor-5 express tendon-specific markers. Tissue Eng Part A 16:2941–2951.

Rees SG, Flannery CR, Little CB, Hughes CE, Caterson B, Dent CM. 2000. Catabolism of aggrecan, decorin and biglycan in tendon. Biochem J 350:181–188.

Rodrigues MT, Reis RL, Gomes ME. 2013. Engineering tendon and ligament tissues: Present developments towards successful clinical products. J Tissue Eng Regen Med 7:673–686.

Saito Y, Yoshizawa F, Takizawa F, Ikegame O, Ishibashi K, Okuda K, Hara K, et al. 2002. A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and runx2/cbfa1/osf2 activity, part of which can be overcome by bone morphogenetic protein-2. J Cell Sci 115:4192–4200.

Schweitzer R, Chyung JH, Mutaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ. 2001. Analysis of the tendon cell fate using scleraxis, a specific marker for tendons and ligaments. Development 128:3855–3866.

Sena K, Morotome Y, Baba O, Terashima T, Takano Y, Ishikawa I. 2003. Gene expression of growth differentiation factors in the developing periodontium of rat molars. J Dent Res 82:166–171.

Seo BM, Miura M, Gronthos S, Bartold PM, Brahim J, Young M, Robey PG, et al. 2004. Investigation of multipotent postnatal stem cell from human periodontal ligament. Lancet 364:149–155.

Settle Jr SH, Rountree RB, Sinha A, Thacker A, Higgins K, Kingsley DM. 2003. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. Dev Biol 254:116–130.

Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. 2005. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthod Craniofac Res 8:191–199.

Shukunami C, Takimoto A, Oro M, Hiraki Y. 2006. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol 298:234–247.

Smith L, Xia Y, Galatz LM, Genin GM, Thomopoulos D. 2012. Tissue-engineering strategies for the tendon/ligament-to-bone insertion. Connect Tissue Res 53:95–105.

Stavropoulos A, Windisch P, Gera I, Capsius B, Sculean A, Wikesjo UM. 2009. A phase IIa randomized controlled clinical and histological pilot study evaluating rhGDF-5/β-TCP for periodontal regeneration. J Clin Periodontol 38:1044–1054.

Stom EE, Kingsley DM. 1996. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 122:3969–3979.

Storm EE, Kingsley DM. 1999. GDF5 coordinates bone and joint formation during digit development. Dev Biol 209:11–27.

Storm EE, Huynh TV, Copeland NG, Jankins NA, Kingsley DM, Lee SJ. 1994. Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 368:639–643.

Sumita Y, Honda MJ, Ueda M, Asahina I, Kagami H. 2010. Differential effects of growth differentiation factor-5 on porcine dental papilla- and follicle-derived cells. Growth Factors 28:56–65.

Tan SL, Ahmad TS, Merican AM, Abbas AA, Ng WM, Kamarul T. 2012. Effect of growth differentiation factor 5 on the proliferation and tenogenic differentiation potential of human mesenchymal stem cells in vitro. Cells Tissues Organs 196:325–338.

Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP. 1996. A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. Nat Genet 12:315–317.

Towler DA, Gelbeman RH. 2006. The alchemy of tendon repair: A primer for the (S)mad scientist. J Clin Invest 116:863–866.

Wang Q, Chen Z, Piao Y. 2005. Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. J Biosci Bioeng 100:418–522.

Wolfman NM, Hattersley G, Cox K, Celeste AJ, Nelson R, Yamaji N, Dube JL. 1997. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, 7, members of the TGF-β gene family. J Clin Invest 100:321–330.

Yoki T, Saito M, Kiyono T, Iseki S, Kosaka K, Nishida E, Tsubakimoto T, et al. 2007. Establishment of immortalized dental follicle cells for generating periodontal ligament in vivo. Cell Tissue Res 327:301–311.

Zhao M, Xiao G, Berry JE, Franceschi RT, Reddi A, Somerman M. 2002. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. J Bone Miner Res 17:144–151.

Supplementary material available online

Appendix Table

For personal use only.

Morphology-Based Prediction of Osteogenic Differentiation Potential of Human Mesenchymal Stem Cells

Fumiko Matsuoka[1], Ichiro Takeuchi[2], Hideki Agata[3], Hideaki Kagami[3,4], Hirofumi Shiono[5], Yasujiro Kiyota[5], Hiroyuki Honda[1], Ryuji Kato[1,6]*

1 Department of Biotechnology, Graduate School of Engineering, Nagoya University, Nagoya, Aichi, Japan, **2** Department of Engineering, Nagoya Institute of Technology, Nagoya, Aichi, Japan, **3** Tissue Engineering Research Group, Division of Molecular Therapy, The Institute of Medical Science The University of Tokyo, Tokyo, Japan, **4** Department of Oral and Maxillofacial Surgery, Matsumoto Dental University School of Dentistry, Shiojiri, Nagano, Japan, **5** Nikon Corporation, Tokyo, Japan, **6** Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya, Aichi, Japan

Abstract

Human bone marrow mesenchymal stem cells (hBMSCs) are widely used cell source for clinical bone regeneration. Achieving the greatest therapeutic effect is dependent on the osteogenic differentiation potential of the stem cells to be implanted. However, there are still no practical methods to characterize such potential non-invasively or previously. Monitoring cellular morphology is a practical and non-invasive approach for evaluating osteogenic potential. Unfortunately, such image-based approaches had been historically qualitative and requiring experienced interpretation. By combining the non-invasive attributes of microscopy with the latest technology allowing higher throughput and quantitative imaging metrics, we studied the applicability of morphometric features to quantitatively predict cellular osteogenic potential. We applied computational machine learning, combining cell morphology features with their corresponding biochemical osteogenic assay results, to develop prediction model of osteogenic differentiation. Using a dataset of 9,990 images automatically acquired by BioStation CT during osteogenic differentiation culture of hBMSCs, 666 morphometric features were extracted as parameters. Two commonly used osteogenic markers, alkaline phosphatase (ALP) activity and calcium deposition were measured experimentally, and used as the true biological differentiation status to validate the prediction accuracy. Using time-course morphological features throughout differentiation culture, the prediction results highly correlated with the experimentally defined differentiation marker values (R>0.89 for both marker predictions). The clinical applicability of our morphology-based prediction was further examined with two scenarios: one using only historical cell images and the other using both historical images together with the patient's own cell images to predict a new patient's cellular potential. The prediction accuracy was found to be greatly enhanced by incorporation of patients' own cell features in the modeling, indicating the practical strategy for clinical usage. Consequently, our results provide strong evidence for the feasibility of using a quantitative time series of phase-contrast cellular morphology for non-invasive cell quality prediction in regenerative medicine.

Citation: Matsuoka F, Takeuchi I, Agata H, Kagami H, Shiono H, et al. (2013) Morphology-Based Prediction of Osteogenic Differentiation Potential of Human Mesenchymal Stem Cells. PLoS ONE 8(2): e55082. doi:10.1371/journal.pone.0055082

Editor: Beatriz Pelacho, Foundation for Applied Medical Research, Spain

Received October 8, 2012; **Accepted** December 18, 2012; **Published** February 21, 2013

Copyright: © 2013 Matsuoka et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: We are grateful to the New Energy and Industrial Technology Development Organization (NEDO) for the Grant for Industrial Technology Research (Financial Support to Young Researchers, 09C46036a) for the support. The research was also supported in part by a grant-in-aid from Nikon Corporation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: We have the following interests. This study was supported in part by a grant-in-aid from Nikon Corporation. Hirofumi Shiono and Yasujiro Kiyota are employed by Nikon Instruments. Ryuji Kato is one of the main investigators of 12 patents (pending) related to basic technologies of "algorithms of cellular image processing. Hirofumi Shiono, is one of the inventors of the hardware we used in our research, "BioStaion CT" which is the automatic cell culture system, and a member of this collaborative project with Nagoya Univ. The BioStation CT and relating software are marketed products of Nikon Corporation. Although the system and the software were used in this study, the result of this research does not depend on the system and the software. He is one of the inventors of related 12 patents. Yasujiro Kiyota, is one of the inventors of the hardware we used in our research work. There are no further patents, products in development or marketed products to declare. This does not alter our adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: kato-r@ps.nagoya-u.ac.jp

These authors contributed equally to this work.

Introduction

Mesenchymal stem cells (MSCs) are a useful cell source for tissue engineering and regenerative medicine of various tissues because of their multi-lineage differentiation capacity (e.g., osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic) [1–3]. Although MSCs can be harvested from various tissues, including adipose tissue and dental pulp, bone marrow derived MSCs (BMSCs) have a well-described *in vivo* bone-forming capacity and are widely used for clinical bone regenerative therapies [4–6]. Several groups, including ours, have been successful in clinical bone tissue engineering using human bone marrow mesenchymal stem cells (hBMSCs) [7–9]. In spite of documented clinical successes of bone regeneration with hBMSCs,

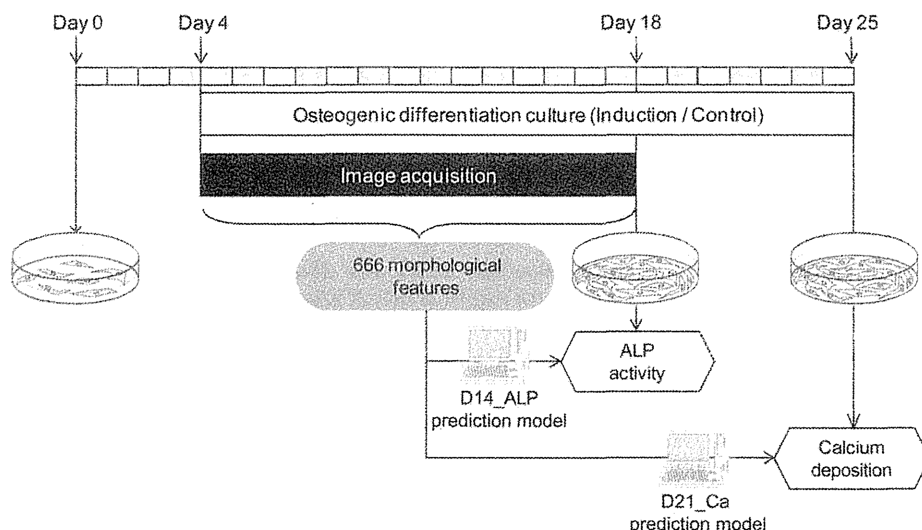


Figure 1. Schematic illustration of the experimental scheme for the prediction of osteogenic differentiation potential using multiple and time-course morphological features. hBMSCs were cultured in non-induction medium in first 4 days, then the medium was replaced with osteogenic induction medium only for the Induction sample. From day 0 to day 14, cell images were automatically acquired by BioStation CT every 8 hours. ALP activity and calcium deposition rates were evaluated on days 14 and 21, respectively. Using multiple morphological features covering 2 weeks culture, two types of hBMSC osteogenic differentiation evaluation results were predicted by individual prediction models.
doi:10.1371/journal.pone.0055082.g001

robust therapeutic efficacy able to withstand the large variation among patients remains a challenge. Therefore, practical and effective cell-quality assurance methods are a necessary approach to address the unmet need of minimizing variability in patient outcomes.

Previous works aimed at characterizing BMSC osteogenic potential have mainly focused on methods that damage cells (e.g., staining, gene expression, etc.) [10]. These conventional techniques limit clinical translation in two ways. First, the destructive nature of the measurements consumes cellular material that would otherwise be useful for therapy. Second, the sample measurements are terminal endpoints, in part due to the irreversible damage incurred by the cells from the measurement procedure. As a result, repeated measurement on the same cellular sample is not possible and longitudinal sampling consumes more material.

Currently, the daily monitoring of cellular morphology by microscopy is combined with minimum sampling for biochemical markers to serve as the routine cellular quality assessment during the expansion culture process. Qualitative microscopic examination and the consumptive nature of the biochemical assays impose a limit on the predictive control currently available in clinical practice. A quantitative, non-invasive method for predicting cellular osteogenic potential and quality is needed to better anticipate clinical outcomes.

Cellular morphology has historically been used as an important indicator to characterize present and assess cell quality. Several reports describe correlations between osteogenic differentiation potential and cellular morphology. Kelly *et al.* have reported that cell geometry is highly correlated with differentiation into osteogenic lineages [11]. Takagi *et al.* have also reported that the cell roundness of hBMSCs is highly correlated with the expression of osteogenic differentiation marker genes [12]. In addition to the above examples that match morphology and cell potential, there are increasing numbers of reports describing image-based cell assessment methods. The popularity of fluorescence-labeled imaging methods in high content cellular screening has outpaced methods with non-labeled image-based assessment; however these approaches retain some technical drawbacks, which do not necessarily improve upon the non-labeled methods [13–16].

In this study, we aimed to demonstrate the efficacy of the non-invasive prediction model, which only uses cellular morphology features to forecast the osteogenic differentiation potential of hBMSCs. Specifically, the outcomes of two biochemical osteogenic markers were quantitatively forecast by two types of prediction models: (1) the alkaline phosphatase (ALP) activity 14 days after differentiation, designated as "D14_ALP model", and (2) the calcium deposition rate 21 days after differentiation, designated as "D21_Ca model". ALP activity is a BMSC differentiation marker; however measuring ALP activity alone is not sufficient for predicting *in vivo* bone formation. Compared to ALP, calcium deposition rate is an osteogenic differentiation marker that highly correlates with *in vivo* bone formation. However, since calcium deposition is a late phenotypic marker, which appears beyond the optimal implantation stage, it is not commonly applied as a clinically useful marker. Overall, it is impossible to measure both markers with the same cell sample or quantitatively predict the measurement results using conventional methods.

To advance this field, we aimed to investigate whether a morphology-based prediction model is capable of quantitatively predicting both ALP activity and calcium deposition rate. Further on, to demonstrate the clinical feasibility of our resulting morphology-based prediction models we examined practical considerations for use in the clinic in order to predict osteogenic potential for new patients scheduled for cell therapy. Two simulation scenarios were carefully examined: (Scenario I) Prediction of osteogenic differentiation potential of a new patient's cells by a model trained with historical patient data; (Scenario II) Prediction of osteogenic differentiation potential of a new patient's cells by a combination of historical patient data and partial culture imaging data from the early stage expansion of the new patient's own cells.

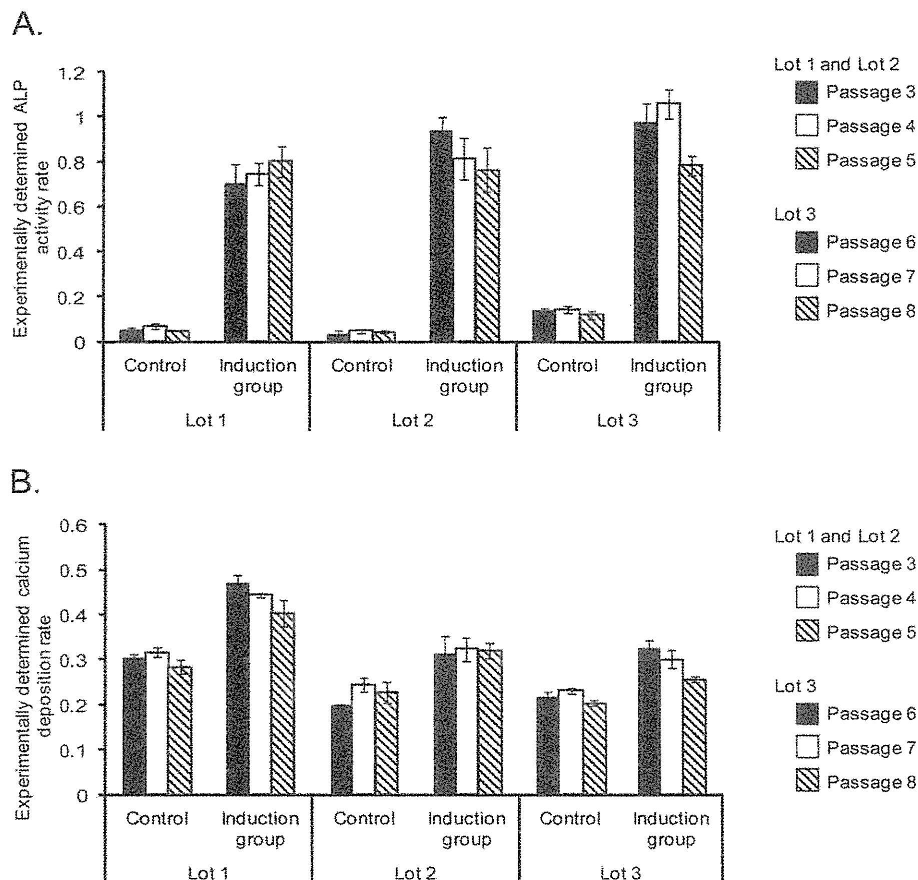


Figure 2. Experimentally determined biological results after the osteogenic differentiation. A: Experimentally determined ALP activity rate on day 14 of differentiation. B: Experimentally determined calcium deposition rate on day 21 of differentiation.
doi:10.1371/journal.pone.0055082.g002

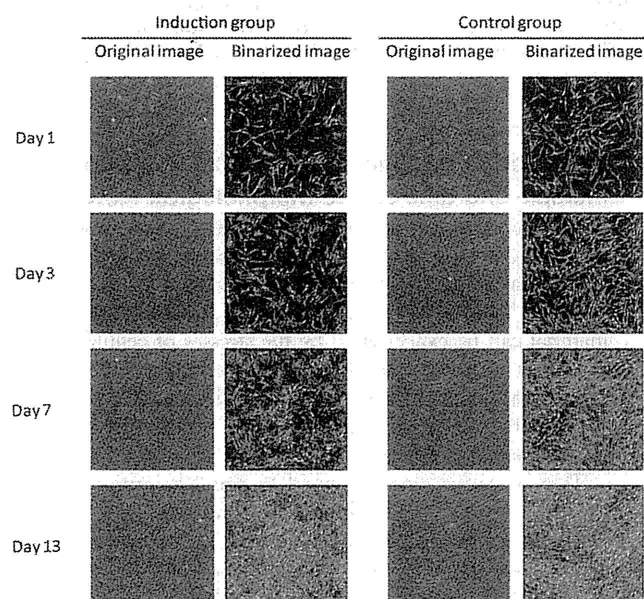


Figure 3. Phase contrast raw image from BioStation CT and its processed image. The images of beginning (day 1), middle (day 3 and 7), and the end (day 13) in the induction period of Lot 1 are indicated as examples. Raw images were binarized with MetaMorph.
doi:10.1371/journal.pone.0055082.g003

Results

Biological/morphological changes during osteogenic differentiation culture

hBMSCs were either cultured in differentiation induction medium or in non-induction medium according to the protocol illustrated in Fig. 1, which was based on the clinical jaw bone therapy protocol used by our group [7]. Three lots of cells, passaged three times per lot, were used to assess "patient-derived variance" and "processing-derived variance." After the image acquisition period during differentiation culture, ALP activity was measured from the same well that the images were acquired. After an additional week of differentiation culture, calcium deposition rate was quantified.

All cell lots at all passages in the induction groups showed a clear increase in ALP activity compared to the control groups (Fig. 2–A). The calcium deposition rate was also significantly higher in the induction group than the control group among all lots and all passages (Fig. 2–B). However, greater variation was observed in the calcium deposition assays compared to the ALP assays. This result reflects the fact that ALP activity measurements add information of osteognic differentiation, but does not qualify as a marker of further osteogenic maturation potential even in *in vitro*.

In contrast to biochemical measurements, which exhibited a noticeable pattern after several weeks of culture, a signature pattern using morphological measurements was found within 7 days of differentiation culture (Fig. 3). For all cell lots at the

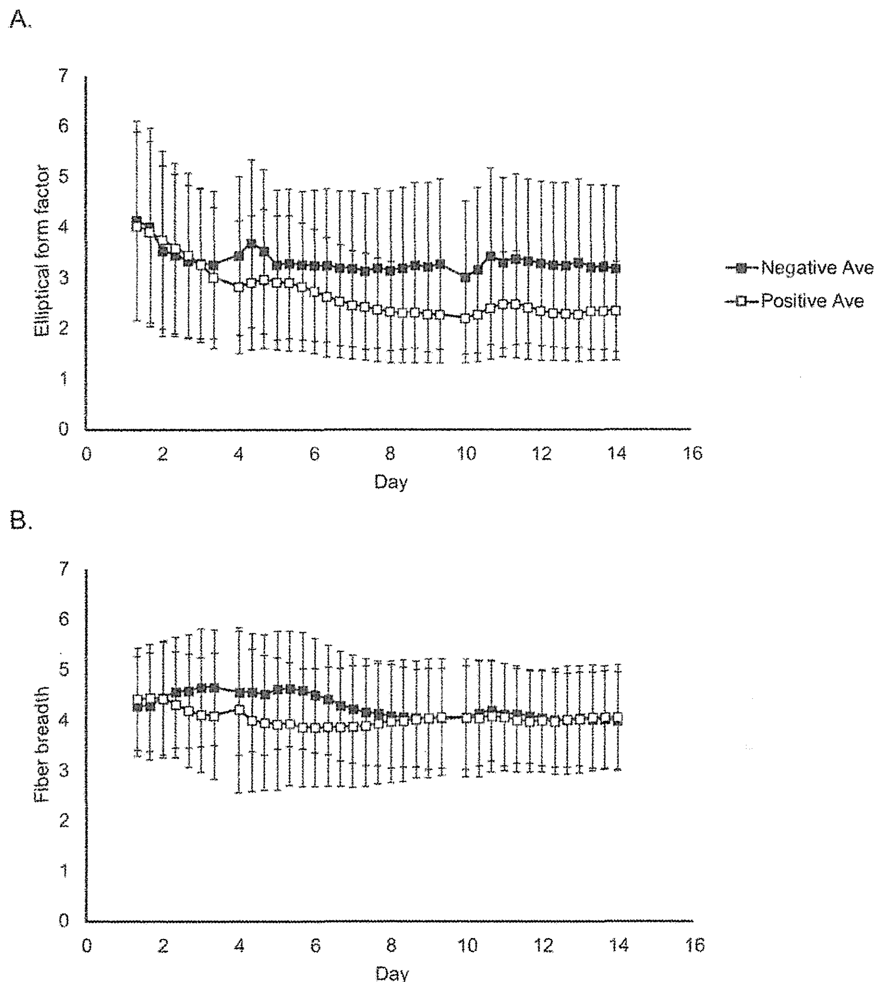


Figure 4. Time series changes of characteristic morphological features. From the 9 morphological features measured, elliptical form factor (A) and fiber breadth (B) of Lot 1 are indicated as representative examples. The symbols indicate the mean value of each morphological feature from all cells in one condition (3 wells ×5 view fields). Roughly, 4,000 to 40,000 cells were measured for the mean. Standard deviations are shown as error bars.
doi:10.1371/journal.pone.0055082.g004

7 day time point, cell morphology in the induction group was observed as flat and spread in multiple two dimensional directions, as compared to the fibroblast-like sharp spindle shape of the control group. By summarizing the quantitative morphological changes in all cells under the same culture conditions through image analysis, an early indication of the cellular phenotype was apparent. For some morphological features, such as Elliptical form factor (the ratio of the object's width to its length) or Fiber breadth (the width of an object modified as a straight fiber), a statistically significant difference ($p<0.01$) between induction and control groups could be identified at a very early culture stage (Fig. 4). Elliptical form factor of Lot 1 was significantly different ($P<0.001$) from day zero at day three of differentiation culture and then throughout the differentiation period. Although these types of morphological differences suggest a relationship to osteogenic induction, they are insufficient to quantitatively predict the final cellular state. To improve predictive power, a machine learning approach was taken to construct a computational model for quantitative prediction and determine the best combination of morphological features to use.

Prediction of osteogenic differentiation potential using multiple and time-course morphological features

Standard practice for bone regeneration therapy is to start by expanding a new patient's cell material to a certain yield, then applies an osteogenic differentiation protocol up until the day of therapy. Variations in the quality of a new patient's starting material can be exacerbated by the stresses of cellular expansion. For these reasons a model for characterizing the regenerative capacity of a patient's cell source, including quality, yield and most importantly osteogenic potential, would add tremendous value to current standard practice.

Two scenarios were designed to simulate anticipated clinical situations available for applying morphology-based prediction models to assess new patient cellular quality. Scenario I: Prediction of new patient BMSC osteogenic potential using a model trained with historical patient data (Fig. 5–A). Scenario II: Prediction of new patient BMSC osteogenic potential using a model trained with historical patient data in addition to data derived from the new patient material (Fig. 5–B). The accuracies of both D14_ALP and D21_Ca models were evaluated in each scenario. Nine morphological features were evaluated from 37 time points over 14 days and complied from 666 image-based input features. The

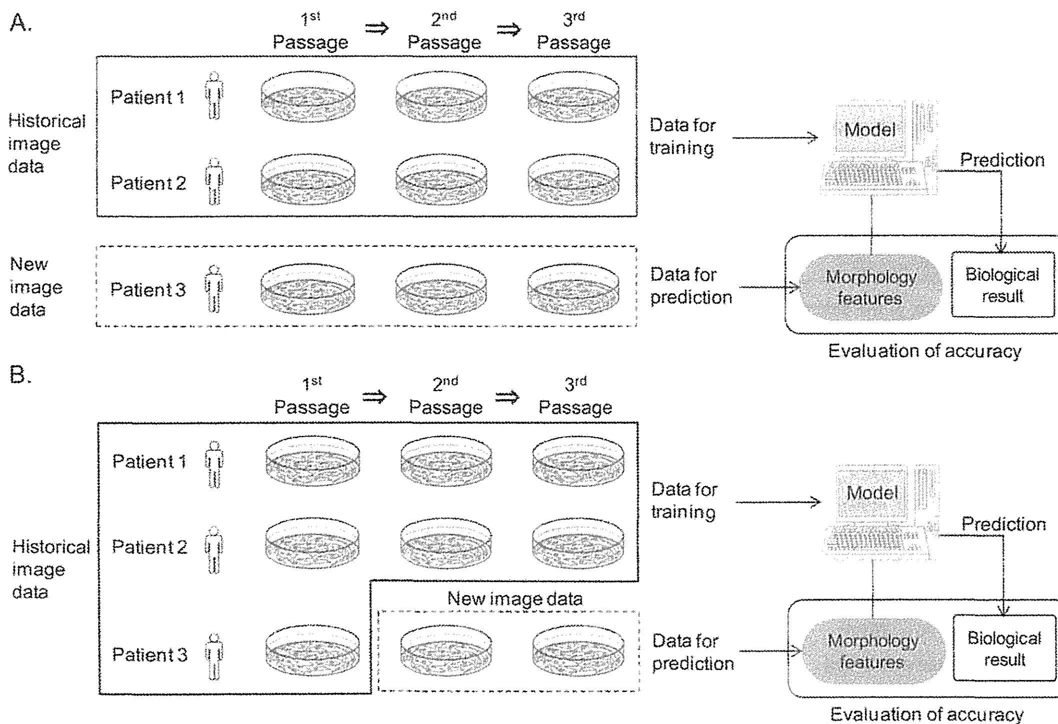


Figure 5. Schematic illustration of two scenarios examined to simulate clinical feasibility. A: (Scenario I) New patient prediction scheme: Trained by historical patient dataset only. Images from all passages of patient 3 were used for prediction. B: (Scenario II) Ongoing patient prediction scheme: Trained by historical patient datasets and a partial dataset from the new patient. For example, for the prediction of cell potential of patient 3, Scheme I uses images of patient 1 and 2 only. Scheme II used images of patient 1 and 2, together with some images from patient 3.
doi:10.1371/journal.pone.0055082.g005

corresponding biochemical differentiation markers from each of the 54 samples were also evaluated. We selected Ridge regression as the machine learning modeling method for linking morphological features to the biomarker measurement results [17]. This method was chosen since Ridge regression is a type of standard regression model that eliminates the multicolinearity problem in multivariate models [18].

Scenario I: New patient prediction scheme. The new patient prediction scheme is designed to simulate the clinical situation where evaluating a new patient's cell quality can be accomplished quickly and reliably (Fig. 5–A). With this scheme, the prediction model can be prepared previously by historical image data from other patients. This model aspires to require no previous data from the new patient.

The prediction accuracies of the D14_ALP and the D21_Ca models are shown in Table 1 and, Fig. 6 (see also Fig. 7 and Table S1 for detailed data). From both prediction results, the correlation coefficients indicated that time-course morphological features of BMSCs during differentiation correlate with the experimentally obtained osteogenic marker values. The average

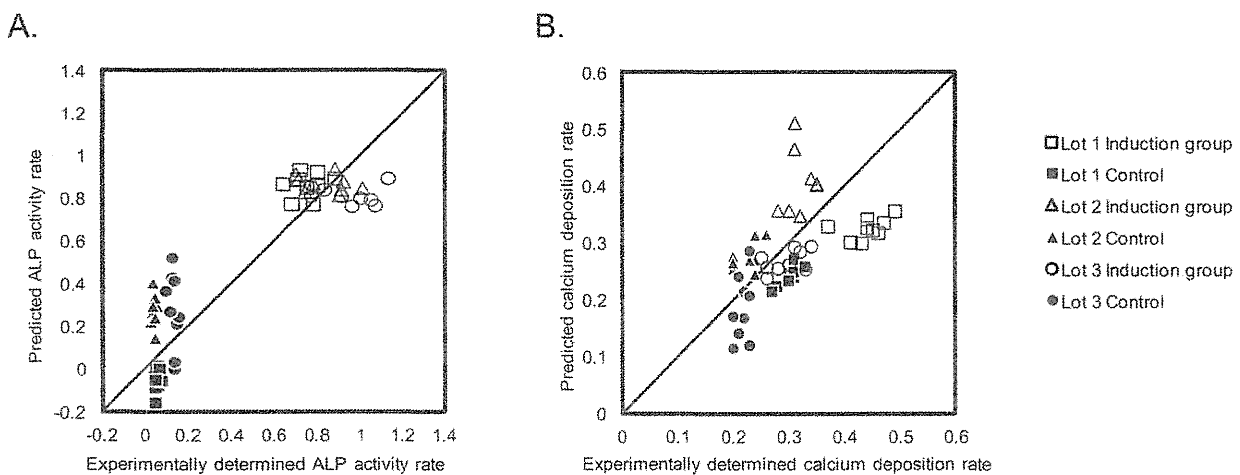


Figure 6. Prediction accuracies in the new patient scheme. A: Scatter plot of experimentally determined values versus predicted values in D14_ALP model, B: Scatter plot of experimentally determined values versus predicted values in D21_Ca model.
doi:10.1371/journal.pone.0055082.g006

Table 1. Prediction accuracy of Ridge regression models for osteogenic differentiation status of hBMSCs.

	New patient prediction scheme		Ongoing patient prediction scheme	
	Ave. absolute prediction error* [−]	R** [−]	Ave. absolute prediction error* [−]	R** [−]
ALP activity rate prediction	0.151	0.903	**0.111**	**0.950**
Calcium deposition rate prediction	0.065	0.526	**0.037**	**0.821**

*Ave. absolute prediction error is the average of the differential between experimentally determined rate and predicted rate.
**Correlation coeffecient between experimentaly determined and predicted rate.
doi:10.1371/journal.pone.0055082.t001

of absolute prediction errors indicates that each prediction model provides predictions within the error rage of ±0.151 with the D14_ALP model and ±0.065 with the D21_Ca model, respectively (Fig. 7). When the variance of all assay data, the result of manual experimental variance, is normalized as 1.0, the prediction errors between different assay measurements can be standardized as 0.194 (D14_ALP) and 0.963 (D21_Ca). This standardized error provides the interpretation that the prediction values are 5-fold stable (D14_ALP) or nearly equal (D21_Ca) compared to the human assay variances.

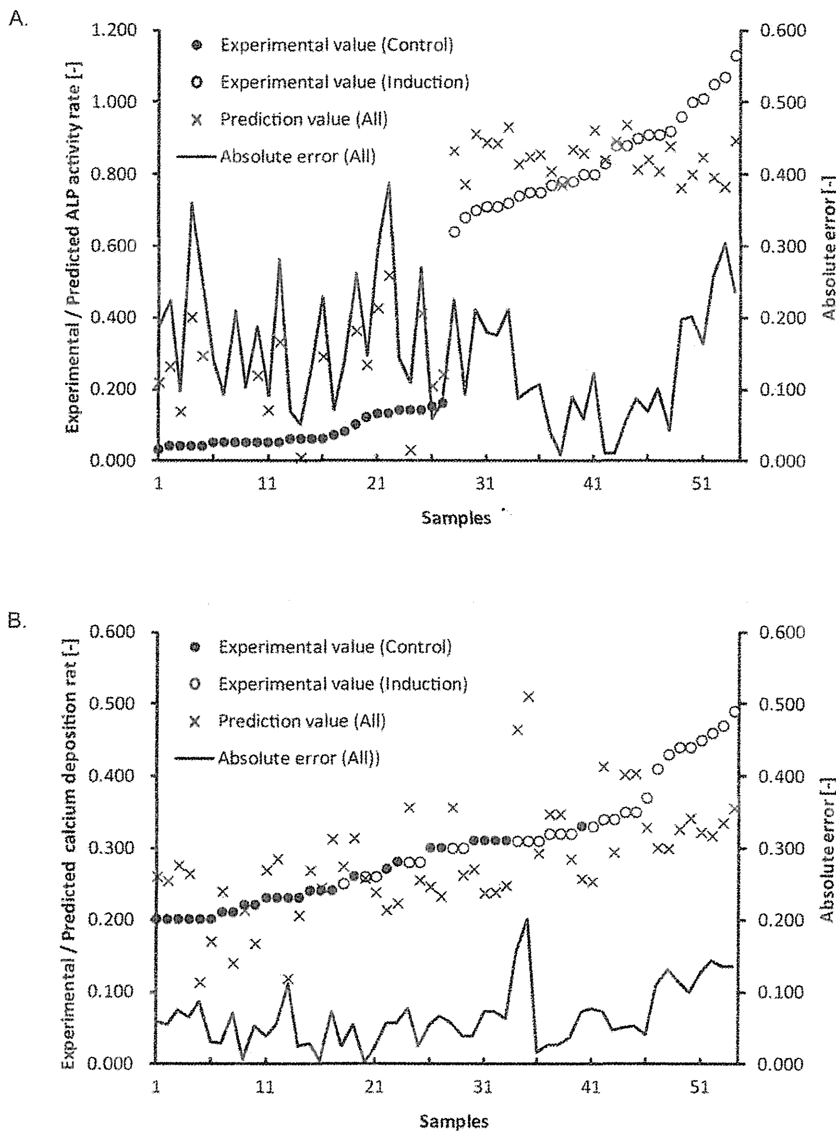


Figure 7. Detailed prediction results in new patient scheme. A: Prediction results and error range in the D14_ALP model. B: Prediction results and error range in the D21_Ca model. All the plotted data were rearranged in the order of experimental values.
doi:10.1371/journal.pone.0055082.g007